ASSAY DEVELOPMENT FOR QUANTIFICATION OF PERSISTERS IN *BURKHOLDERIA CENOCEPACIA* BIOFILMS

Jenni Sarekoski
University of Helsinki
Faculty of Pharmacy
Division of pharmaceutical biology
January 2018

HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Faculty	Department
Faculty of Pharmacy	Division of Pharmaceutical Biosciences

Author

Jenni Sarekoski

Title

Assay development for quantification of persister cells in *Burkholderia cenocepacia* biofilms

Subject

Pharmaceutical Biology

Level	Month and year	Number of pages
Master's thesis	January 2018	48

Abstract

Most bacteria live as biofilms (99%), which is a population of cells attached to a natural or artificial surface and encased in self-produced exopolysaccharide matrix. The extracellular polymeric substances (EPS) in the matrix can vary greatly between species in chemical and physical properties, but primarily it consists of water, polysaccharides, proteins, nucleic acids and absorbed nutrients from the surrounding area. Biofilm formation appears to be a survival strategy of bacteria and the main purpose of the biofilm matrix is to protect the bacteria. In nature, biofilms have been found in variety of different environments, including humans.

Bacterial biofilms demonstrate a decreased susceptibility to antimicrobial agents and several mechanisms have been proposed to be involved in this tolerance. One of the reasons why chronic infections develop is that the immune response fails to remove the biofilm. Most of the bacterial infections currently in developed countries are biofilm related and these infections are often recalcitrant and difficult to eradicate with available treatments. In addition to chronic infections, the treatment of acute infections is shadowed by increasing problems with highly resistant bacteria.

The presence of dormant persisters in biofilms accounts for their tolerance to antimicrobials and likely are responsible for latent and chronic infections, such as tuberculosis. Persistence is not primarily an active mechanism of antibiotic tolerance, but a dormant state of the bacteria avoiding the mechanism of action of most antibiotics. Persisters form stochastically only in small numbers, and more relevant physiological explanation is related to the stress responses of the cells. Persisters are distinguish phenotypic variants of the normal population and it is not a heritable feature, as no mutations occur.

The dormant, persistent state of the bacteria is largely responsible for the multidrug tolerance of recalcitrant infections. Biofilm cause various diseases in humans, as bacteria are able to attach to practically any surface, such as teeth, heart valves, lungs, middle ear, artificial prosthetics and instruments. Biofilms growing on prosthetic joints can cause also serious infections, which are painful for the patient with high risks for complications, expensive and laborious to replace. Biofilm infections are difficult to treat and a huge burden in the healthcare. Many acute infections can be cured with conventional antibiotic therapies, but this is not case with recalcitrant, chronic infections.

B. cenocepacia belongs to the *B. cepacia* complex (Bcc) which consist of 20 closely related and phenotypically similar species. This species was chosen for this study because of its natural tolerance to antibiotics and ability to form biofilms easily. This species causes fatal lung infections in cystic fibrosis patients, and there is no treatment for it other than inadequate combination antibiotic treatment and lung transplant.

In this thesis, a promising method was developed and validated for detecting anti-persister activity against *B. cenocepacia*. The assay is based on measuring the levels of ATP present in the cultures after treatment and it can be used quantify remaining persisters using *B. cenocepacia* biofilms. Utilizing the method validated, it was confirmed that mitomycin C is an effective anti-persister compound against highly tolerant *B. cenocepacia* biofilms even at low concentrations. Doxycycline was found to be ineffective against *B. cenocepacia* biofilms, although the bacteria are susceptible to it in planktonic form, and ciprofloxacin was proved to be effective at very high concentrations.

Keywords

Biofilm, persister, multi-drug tolerance, assay development

Where deposited

The Faculty of Pharmacy, University of Helsinki

Additional information

Thesis supervisor: Doctor Malena Skogman

HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Tiedekunta	Osasto
Farmasian tiedekunta	Farmaseuttisten biotieteiden osasto

Tekijä

Jenni Sarekoski

Työn nimi

Assay development for quantification of persister cells in *Burkholderia cenocepacia* biofilms

Oppiaine

Farmaseuttinen biologia

Työn laji	Aika	Sivumäärä
Pro gradu -tutkielma	Tammikuu 2018	48

Tiivistelmä

Suurin osa bakteereista elää biofilmeinä (99%), eli populaationa soluja kiinnittyneenä luonnolliseen tai keinotekoiseen pintaan. Bakteeripopulaatio kätkeytyy itsetuotettuun eksopolysakkaridi limaan. Solun ulkopuoliset polymeeriset yhdisteet voivat vaihdella suuresti eri lajien välillä kemiallisilta ja fysikaalisilta ominaisuuksiltaan. Pääasiassa kuitenkin tämä lima koostuu vedestä, polysakkarideista, proteiineista, nukleiinihapoista ja ympäristöstä saaduista ravinteista. Biofilmin muodostus vaikuttaa olevan pääasiassa selviytymismekanismi bakteereille, ja biofilmin liman päätarkoitus onkin suojella bakteereita. Luonnossa biofilmejä esiintyy laajalti erilaisissa ympäristöissä, jopa ihmisessä. Bakteerien muodostamat biofilmit osoittavat heikentynyttä herkkyyttä antibiooteille ja useita mekanismeja tämän toleranssin taustalla on selvitetty. Kroonisia infektioita kehittyy mm. seurauksena siitä, että immuunipuolustus epäonnistuu tuhoamaan biofilmin. Suurin osa bakteeri-infektioista kehittyneissä maissa ovat biofilmien aiheuttamia ja nämä infektiot ovat usein hoitoon vastaamattomia nykyisillä hoidoilla. Kroonisten infektioiden lisäksi terveydenhuollon ongelmana on akuuttejakin infektioita aiheuttavien bakteerien kasvava resistenssi antibiooteille.

Uinuvat persisterit biofilmeissä aiheuttavat niiden toleranssiin antimikrobeja vastaan ja persisterien ajatellaankin olevan syynä latentteihin ja kroonisiin infektioihin, kuten tuberkuloosiin. Persistenssi ei ole aktiivinen antibioottitoleranssin mekanismi, vaan ennemmin uinuva bakteerien tila, joka välttää useimpien antibioottien vaikutusmekanismin. Persistereitä muodostuu sattumanvaraisesti vain vähän ja oleellisempi fysiologinen selitys liittyy solujen stressireaktioon. Persisterit ovat erillinen fenotyyppi normaalista populaatiosta, eikä se ole periytyvää, sillä mutaatioita ei tapahdu.

Bakteerien persistentti tila on suuresti vastuussa hoitoon vastaamattomien infektioiden monilääketoleranssista. Biofilmit aiheuttavat erilaisia sairauksia ihmisissä, sillä bakteerit voivat kiinnittyä lähes mihin tahansa pintaan, kuten hampaisiin, sydänläppiin, keuhkoihin, välikorvaan, keinoproteeseihin, ja instrumentteihin. Biofilmien aiheuttamat infektiot ovat vaikeita hoitaa, ja ne ovat suuri taakka terveydenhuollolle. Keinonivelissä kasvavat biofilmit voivat aiheuttaa vakavia infektioita, jotka ovat kivuliaita potilaalle, ja niissä on suuri komplikaatioiden riski. Keinonivelen infektiot tulevat kalliiksi ja työläiksi hoitaa, sillä paras hoito on usein sen vaihtaminen uuteen. *B. cenocepacia* kuuluu *B. cepacia* kompleksiin, joka koostuu 20:sta fenotyypiltään läheisestä lajista. Tämä laji valittiin tähän tutkimukseen sen luonnollisen toleranssin ja sen hyvän biofilmin muodostuskyvyn takia. Laji aiheuttaa fataaleja keuhkoinfektioita potilailla, joilla on kystinen fibroosi. Näihin infektioihin ei ole mitään hoitoa, riittämättömän antibioottihoidon ja keuhkosiirteen lisäksi.

Tässä tutkielmassa kehitettiin ja validoitiin lupaava menetelmä persisterien vastaisen aktiivisuuden havaitsemiseksi *B. cenocepacia* biofilmejä vastaan. Menetelmä perustuu ATP pitoisuuksien mittaamiseen viljelmistä käsittelyn jälkeen. Menetelmää voidaan käyttää käsittelyn jälkeen jäljelle jääneiden persisterien määrän määrittämiseen käyttäen *B. cenocepacia* biofilmejä. Kehitettyä metodia käyttämällä vahvistettiin, että mitomysiini C on tehokas persisterejä tappava yhdiste tolerantteja *B. cenocepacia* biofilmejä vastaan myös erittäin pienillä pitoisuuksilla. Doksisykliini osoittautui tehottomaksi näitä biofilmejä vastaan, vaikka se oli tehokas planktonisessa muodossa kasvaviin bakteereihin. Siprofloksasiini oli tehokas biofilmejä vastaan vain erittäin korkeilla pitoisuuksilla.

Avainsanat

Biofilmi, persisteri, monilääketoleranssi, menetelmän kehitys

Säilytyspaikka

Farmasian tiedekunta, Helsingin yliopisto

Muita tietoja

Työn ohjaaja: Malena Skogman, FT

ACKNOWLEDGEMENTS

This thesis concludes my M. Sc. degree of Pharmacy in the Faculty of Pharmacy of the University of Helsinki. The research work was performed at the Division of Pharmaceutical Biosciences in the Faculty of Pharmacy.

I would first like to express my gratitude to my supervisor Doctor Malena Skogman for the support, guidance and engagement through the process of this thesis. I would like to thank Heikki Vuorela for kindly reviewing the work. I am also grateful for Adjunct Professor Adyary Fallarero for providing valuable feedback on the thesis and drawing my attention to the subject. I would like to honor the memory of Professor Pia Vuorela, who recently passed away. Her enthusiasm and determination in developing the education of pharmacy will not be forgotten.

Furthermore, I thank Ilkka Miettinen for the introduction to the subject and for his enthusiastic way of solving problems. Also, I like to thank Paola and Krista for help on the way, and Eveliina for peer support.

I am grateful for my fellow M. Sc. students of cursus MMXII for endless peer support. I would also like to thank my loved ones, who have supported me throughout entire process. This accomplishment would not have been possible without them. Thank you.

Helsinki, January 6th 2018
Jenni Sarekoski

LIST OF ABBREVIATIONS

(p)ppGpp	guanosine tetra- or pentaphosphate
96-MWP	96-microwell plate
ATP	adenosine triphosphate
CF	cystic fibrosis
CFU	colony forming units
DHA	dehydroabietic acid
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substance
HTS	high-throughput screening
LB	Luria Bertani
PBS	phosphate buffered saline
PMN	polymorphonuclear leukocytes
QS	quorum sensing
RFU	relative fluorescence units
Rpm	rounds per minute
RSD	relative standard deviation
S/B	signal-to-background
S/N	signal-to-noise
TA	toxin-antitoxin
VBNC	viable but non culturable

TABLE OF CONTENTS

1 INTRODUCTION

Biofilms are present in more than 80 % of microbial infections according to the U.S. National Institutes of Health in 2015 (Donne and Dewilde 2015). There are 17 million new biofilm related infections every year in the United States of America, which lead to 550 000 fatalities. Biofilm infections are difficult to treat and a huge burden in the healthcare (Bryers 2008). Many acute infections can be cured with conventional antibiotic therapies, but this is not case with recalcitrant, chronic infections (Alhede et al. 2014). Antibiotics can help the symptoms of a chronic infection and keep the acute phase in control, but as soon as the treatment is halted, the infection might resuscitate. This is mainly because of persisters, highly tolerant bacteria inside the biofilms. Dormant and non-dividing persisters are not affected by traditional antibiotics, which are primarily effective against growing cells.

While resistance is a rising problem in the spotlight, and the need for novel antimicrobial therapies is obvious, tolerance is rarely discussed in such volume. As new antimicrobials are not frequently discovered, the need for novel targets, that would not be sensitive to resistance is evident. New targets like persisters or features contributing to the biofilm formation and intercell communication would be potential targets for novel antimicrobial therapies. Yet, persisters are not a new issue, as they were discovered in the 1940's. Quite little is known of this phenotype, and there is urge for more knowledge. Persisters are rather difficult to study, and many different insights to the issue have been presented, with a few misperceptions (Kim and Wood 2016). The aim of this thesis was to find and validate a method for screening new compounds with anti-persisters activity.

2 LITERATURE REVIEW

2.1 Biofilms

Biofilm is the most common lifestyle for bacteria, and it is estimated that 99% of all bacteria live in biofilms (Donne and Dewilde 2015). Biofilms consist of two main components: the extracellular matrix (80-85%) and only 15-20% of it is bacteria or other microorganisms (Dufour et al. 2010). Biofilms can be formed by a single species, but also various species of bacteria can form a mixed biofilm. In addition to bacteria, also fungi, algae, yeasts and protozoa can form biofilms. In 1933 Henrici made an observation of this bacterial lifestyle and reported that "It is quite evident that for the most part the water bacteria are not free-floating organisms, but grow on submerged surfaces; they are of the benthos rather than the plankton" (Henrici 1933).

Biofilm is a population of cells attached to a natural or artificial surface and encased in self-produced exopolysaccharide matrix (Dufour et al. 2010; Lewis 2001). Biofilms are difficult to eradicate and cause many recalcitrant infections. In nature, biofilms have been found in variety of different environments e. g. thermal waters and frozen glaciers. In addition, biofilms can be found in extreme acidic environments with high metal content and low nutrients, and they can also thrive in very different hydration levels ranging from deserts to oceans (Dufour et al. 2010). Biofilms can grow in abiotic or living surfaces, also inside humans, and pathogenic biofilms are a relevant topic in the healthcare. Most of the bacterial infections currently in developed countries are biofilm related and these infections are often recalcitrant and difficult to eradicate with available treatments (Høiby et al. 2010). In addition to chronic infections, the treatment of acute infections is shadowed by increasing problems with highly resistant bacteria.

Apart from the notorious pathogenic reputation of biofilms, some biofilms are essential to humans, taking part in many vital processes e.g. in the production of vitamins and essential amino acids (Donlan 2002). Useful biofilms can also help prevent colonization by exogenous pathogens. There are also many advantageous roles for biofilms in the environment, e. g. functioning as symbiotic nitrogen fixer in plant roots.

2.1.1 Biofilm formation and structure

Biofilms can be formed practically on any surface. There are certain key steps recognized in the process of biofilm formation. The first stage of biofilm formation is the initial attachment. In addition to passive movements, free-floating bacteria are able to swim towards favourable conditions with e. g. flagellum machinery and attach to surfaces (Beloin et al. 2008; Donne and Dewilde 2015). Physicochemical properties of the surface and the surrounding environment may exert a strong influence on the rate and extent of attachment (Donlan 2002; Purevdorj et al. 2002). Bacteria are able to overcome repulsive forces with flagella or pili, or there can exist hydrophobic or non-polar interactions between the cell surface and the substratum which enhance the irreversible attachment. When any surface is exposed to aqueous medium it will inevitably become conditioned or coated with polymers from that medium. These conditioning films will influence also the hydrodynamics and thus the attachment of bacteria to materials (Donlan 2002).

Perhaps surprisingly, it has been observed that bacteria form biofilms preferably in conditions with high shear forces (Donlan 2002) . This can though, be explained by the hydrodynamics boundary layer, which substantially affects the interactions between cell surface and the substratum. The velocity characteristics of the liquid will influence the rate of settling of the bacteria and association of cells with a surface (Rijnaarts et al. 1993; Zheng et al. 1994). Planktonic bacteria behave as particles in liquid and under very low linear velocities the bacteria must cross broad hydrodynamic boundary layers, and contact with the surface would require high cell motility. As the velocity increases, the boundary layer decreases and the bacteria are exposed to greater turbulence and mixing. This is true until the velocities become high enough to exert shear forces to the attaching cells, and detaching them from the surface. Also, the properties of the medium (nutrients etc.) affect the attachment of the bacteria and growth of the biofilm (Donlan 2002; Purevdorj et al. 2002). External physiochemical factors can affect the gene expression in biofilms, and attached cells may be involved in gene regulation of other cells by complex regulatory pathways (Donlan 2002). Along with environmental factors, properties of the microbial cell, presence of fimbriae, flagella or pili and production of surface associated polysaccharides or proteins all influence the rate and extent of attachment.

Biofilm can be flat or mushroom shaped, depending on the environment, nutrient source and the motility machinery present (Hall-Stoodley et al. 2004; Patriquin et al. 2007). Low oxygen, limitations in iron ions or sub lethal concentrations of antibiotics can enhance biofilm formation (Götz 2002; Patriquin et al. 2007). The extracellular polymeric substances (EPS) in the matrix can vary greatly between species in chemical and physical properties, but primarily it consists of water, polysaccharides, proteins, nucleic acids and absorbed nutrients from the surrounding area. EPS account for 50-90% of the organic carbon of biofilms. Biofilm formation appears to be a survival strategy of bacteria and the main purpose of the biofilm matrix is to protect the bacteria (O'Toole et al. 2000). The matrix composition is extremely important for the properties of the biofilm and it gives protection from various environmental challenges like antibiotics or host's immune system (Gunn et al. 2016). Bacteria can live also in a form similar to biofilm, as aggregates, when bacteria attach to each other only, and no surface is involved (Alhede et al. 2011).

There are multiple strategies for the bacteria to detach from the biofilm (Donlan 2002). Biofilm cells may shed as daughter cells of actively growing cells, detach as a result of changes in the environment, due to quorum sensing signals (or other signals) or shearing of biofilm aggregates because of shear stress. Many observations from the nature can be applied to situations in humans, as basics of biofilm growth and dispersal are alike (Hall-Stoodley et al. 2004). Although mixed-species biofilms thrive in most natural environments, single-species biofilms cause in a variety of infections and live on the surface of medical implants.

2.1.2 Biofilm as a bacterial lifestyle

Both the environment and genes play important and complex roles in biofilm development (Hall-Stoodley et al. 2004). The proximity of cells inside the biofilm or aggregates provide an ideal opportunity for gene exchange and bacterial communication. To adapt to the changes of the environment, bacteria are able to utilize this specific mechanism of bacterial communication. Bacteria cells emit and detect small diffusing compounds to evaluate the population's size and to express specific genes according to the population size and the environment (Brackman et al. 2009). These diffusible signal molecules are produced constantly, but at a low population density they provoke no effect.

This kind of intercellular communication between bacteria is called quorum sensing (QS). When population density is sufficiently high, the signal molecules bind to the receptors inducing or repressing transcription of QS-regulated genes. As an example, gram-negative species belonging to the family of *Burkholderia* utilize multiple QS systems such as N-acyl-homoserine lactone (AHL)-dependent system for intercell communication (Schmid et al. 2012; Sokol et al. 2007). With QS, bacteria can adapt to different levels of nutrients in the environment, defend against competitive microorganisms and avoid toxic materials (Galloway et al. 2011). QS is also needed for invading or infecting a host.

QS is not the sole mechanism responsible for biofilm formation and the virulence of the bacteria, there are other signals from the environment that can control biofilm formation and structure as well (Purevdorj et al. 2002). Although QS is a promising target for new antimicrobials, these kinds of compounds seem to mostly just enhance the effect of other antimicrobials, by weakening the biofilm (Brackman et al. 2009). There are many other pathways included in the biofilm formation and interfering with QS does not completely block the biofilm from forming, but might have a weakening effect. This might be useful, when therapies are combined with antibiotics. One benefit in targeting the QS as an anti-biofilm tactic is that QS inhibitors are typically used in concentrations below the minimal inhibitory concentration (MIC) and thus it is less likely that they would impose a selective pressure for the development of resistance.

Biofilm formation and surface adhesion provide many advantages for the bacteria, tolerance and virulence of biofilms are characteristic to the lifestyle (Hall-Stoodley et al. 2004). The matrix of the biofilm can protect the bacteria from antimicrobials by slowing physically the penetration of antibiotic or bind antibiotics directly to the EPS (Donlan 2002). Shiau and Wu (1998) showed that the extracellular polymeric substance matrix produced by *Staphylococcus epidermidis* interfered with macrophage phagocytic activity. The biofilm matrix provides also protection from immune system, and can slow the penetration of some antibiotics by negatively charged polymers (Fux et al. 2005). A solid surface provides also stability in the growth environment. Biofilm present a selective barrier for the immune system, and it has been also shown that human leukocytes are able to penetrate the biofilm, but unable to engulf the bacteria present in the biofilm (Leid et al. 2002). This suggests that there is another mechanism that inhibits normal leukocyte function within the biofilm. Leukocytes can possibly penetrate the biofilm through

nutrient and flow channels present in the biofilm (Bjarnsholt et al. 2005). This supports the observation that biofilms are more like extremely porous hydrogels than solid rigid structure.

2.1.3 Biofilm recalcitrance

Bacterial biofilms demonstrate a decreased susceptibility to antimicrobial agents and several mechanisms have been proposed to be involved in this tolerance. One of the reasons why chronic infections develop is that the immune response fails to eradicate the biofilm. This deficiency appears to be independent of the localization of the biofilm in the host (Bjarnsholt et al. 2009).

It has been suggested that antibiotic treatment and the presence of host's immune cells induces defence mechanism in biofilms (Jensen et al. 2007). The polymorphonuclear leukocytes (PMNs) constitute the most abundant of the circulating leukocytes that are normally the first phagocytes to appear in high numbers at the site of infections. The major role of the PMNs in acute inflammation is to phagocytize microorganisms and foreign materials (Bjarnsholt et al. 2005). The QS controlled production of rhamnolipids by the bacteria have been shown to disable the PMNs in *Pseudomonas aeruginosa* (Jensen et al. 2007). A range of antibiotics have been examined for their effects on QS-regulated virulence factors like rhamnolipids, and many conventional antibiotics have shown to exert strong QS-inhibitory activity in *P. aeruginosa* (Skindersoe et al. 2008).

Efflux pumps are involved in bacterial multidrug resistance, but it is not completely clear if effluxes are also involved in the mechanism of tolerance (Van Acker and Coenye 2016). It is known that inhibiting efflux pumps weakens the biofilm and may block maturation of biofilm (Baugh et al. 2014; Kvist et al. 2008). This might influence the virulence of the bacteria, since virulence is linked to the ability to form biofilm. However, it seems that the connection of efflux pumps to resistance is more relevant than the link to persistence (Kim and Wood 2016). Both phenomena tolerance and resistance are clinically relevant in chronic infections, though they are not related my mechanism.

It appears that another main immune defence, the production of reactive oxygen species (ROS), has a side effect of activating both classical resistance and multidrug tolerance in

pathogen cells (Wu et al. 2012). ROS induce oxidative stress, but by doing that, the concentration of antibiotics may decrease in the cell increasing the level of surviving persisters and their tolerance to this and other antibiotics, worsening recalcitrance.

There are several features in the biofilm recalcitrance, but it seems that the main reason behind the tolerance lays in the dormant phenotypic variants of the normal population (Lewis 2010). Because antibiotics kill cells by corrupting specific targets in actively dividing and growing cells, dormant cells, in which the antibiotic targets are inactive, avoid killing (Wu et al. 2012). These non-growing, dormant cells expressing the specific temporary phenotype are called persisters.

2.2 Persister cells

Persister cells were first described in one of the first studies on the mechanism of penicillin action by Joseph W. Bigger in 1944 (Bigger 1944). In the experiment, Bigger discovered that a *Staphylococcus aureus* culture killed with penicillin could regrow in the absence of the antibiotic. This new culture could be lysed again with penicillin, yet again, a small subculture survived. These surviving cells were named as "persisters" to differentiate them from resistant bacteria, which can grow in the presence of the antibiotic. The tolerance of persisters to antibiotics is non-heritable and reversible (Keren et al. 2004). Although persisters were discovered right after the discovery of antibiotics, they were not studied for almost four decades. The main reason why persister were forgotten after their discovery was the tiny fraction of persisters in cultures and difficulty to isolate them with the techniques available at the time.

In the 80's Moeyd discovered high persister mutants (*hip*) of *Escheria coli* and this discovery attracted attention to the subject again (Moyed and Bertrand 1983). It was not until a decade later when interest in persisters was notably increasing after the discovery of these high persister mutants. In the 1990's resistance was developing faster than the discovery of new antibiotics and chronic infections were on the rise due to increasing use of indwelling devices and the rise in immunocompromised patients owing to cancer chemotherapy and HIV (Lewis 2010).

Persister cells are dormant phenotypic variants of regular cells and are highly tolerant to antibiotics (Wu et al. 2012). Cells can enter this dormant state spontaneously or through different mechanisms induced by the environment (Kwan et al. 2013). Persisters neither die or nor grow in the presence of antibiotics because antibiotics are unable to disturb cellular processes in a cell with globally reduced metabolism. The dormant, persistent state of the bacteria is largely responsible for the multidrug tolerance of recalcitrant infections. Chronic infections can be typically restrained but not eradicated completely with existing antimicrobials. The presence of dormant persisters in biofilms accounts for their tolerance to antimicrobials and likely are responsible for latent and chronic infections, such as tuberculosis.

Persisters are found in all phases of cell growth, with a frequency of 0.0001 to 0.001% of the population in exponential-phase cultures and as high as 1% in biofilms and stationary-phase cultures (Kwan et al. 2013; Shah et al. 2006). Persisters are distinguish phenotypic variants of the normal population and no mutations occur, thus it is not a heritable feature (Figure 1) (Keren et al. 2004). It is however important to notice that non-dividing cells in stationary phase are not persisters, even though they behave like persisters (Lewis 2007). These non-persister cells will start dividing soon although they are not dividing at the moment of measurement.

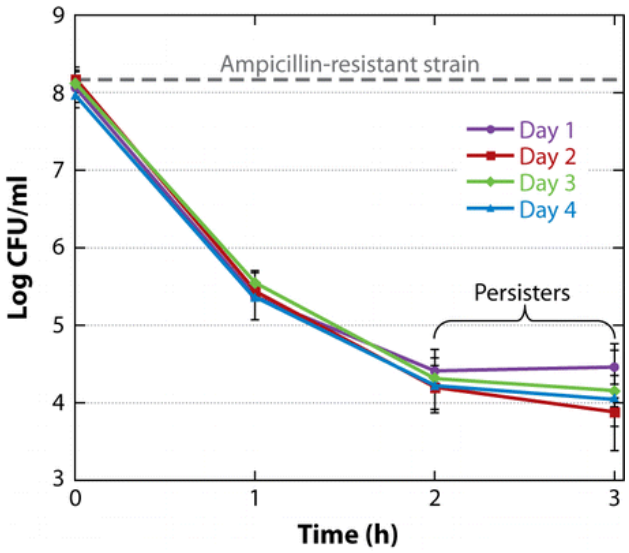

Figure 1. Keren et al. (2004) repeated the experiment made by Bigger in 1944, where a bacterial culture was treated with ampicillin several times after reviving it in the absence of the antibiotic, and the strain remains sensitive to the antibiotic, behaving differently than the resistant strain.

2.2.1 Mechanism of persister formation

Persistence is not primarily an active mechanism of antibiotic tolerance, but a dormant state of the bacteria avoiding the mechanism of action of most antibiotics (Wood et al. 2013). Various descriptions of persister cell characteristics and for mechanism of persister formation have been proposed, but foremost persisters are dormant cells that halt protein synthesis (Kim and Wood 2016; Kwan et al. 2013). The proposed mechanisms leading to arrested protein synthesis include toxin-antitoxin modules, stringent response through the alarmone guanosine penta- or tetraphosphate (p)ppGpp, and to some extent spontaneous formation (Maisonneuve and Gerdes 2014). Lack of nutrients like amino acids and other environmental cues increase persister cell formation (Kwan et al. 2013). Persisters form stochastically only in small numbers, and more relevant physiological explanation is related to the stress responses of the cells. Expression of the SOS response to (deoxyribonucleic acid) DNA damage by fluoroquinolone treatment induces formation of persisters by increasing expression of a specific toxin. Also, induction of a classical resistance mechanism, multidrug-resistant (MDR) efflux pump, by oxidative stress leads to an increase in persister cells (Wu et al. 2012).

One of most studied mechanism for persister formation is toxin-antitoxin (TA) modules (Lewis 2010). Typically, the toxin part of the module is a protein that inhibits an important cellular function. The antitoxin counterpart forms an inactive complex with the toxin, and no damage to translation or replication occurs. Under specific stresses, antitoxins are degraded and the resulting active toxins inhibit cellular processes, which eventually leads to persister formation (Gerdes and Maisonneuve 2012). TA modules were first linked to persisters in 2006 (Shah et al. 2006). TA modules are prevalent in bacterial genome and they play an important role in persister formation (Gerdes and Maisonneuve 2012). To activate the TA systems leading to persistence, the cell must respond to stress (Korch et al. 2003). This stress response is thought to be propagated through the alarmone guanosine tetraphosphate (ppGpp) which is produced during nutrient limitation (stringent response) and other stresses (Maisonneuve et al. 2013). It has been suggested that the ppGpp mediating the stringent response activates Lon protease through polyphosphate, and subsequently Lon inactivates antitoxins of TA systems freeing the toxin to reduce metabolism. PpGpp has been linked to persistence before, but Maisonneuve et al. (2013) made conclusion that the ppGpp activates Lon protease via polyphosphate (Korch et al.

2003). However, there is a controversy to this conclusion, as it has been showed that ppGpp and polyphosphate rather inactivate Lon protease (Osbourne et al. 2014). In cells unable to produce ppGpp, persister cells are still formed, so there must be other mechanism for persister formation (Chowdhury et al. 2016). It has been also studied that many TA systems were still able to increase persistence in the absence of ppGpp.

Although many mechanisms behind persistence has been studied, the connection between an identified mechanism and persister formation seem to be controversial, and there are quality and consistency issues in many studies (Kim and Wood 2016). This proves that persistence is still today a difficult subject to study, and the basic methods need validation. The study of anti-persisters could be a straight-forward continuum to the studies of antimicrobials, but the insights into the mechanism leading to persistence are varying and the complexity of the studies is increased since there is not one protein responsible for the phenomenon (Chowdhury et al. 2016). Also, there are many errors abound in the techniques studying persisters with many waking the cells with fresh medium leading to the activation of the phenotype, and non-persister cells are studied eventually (Kim and Wood 2016). As microbial infections are the leading cause of death worldwide and persister cells being the main reason for recurring infections, the need for more accurate details for the mechanism of persister cell formation is evident (Lewis 2007; Rasko and Sperandio 2010).

2.2.2 Medical challenge of tolerant persisters

Treatment of bacterial infections is distracted by the two distinct phenomena of bacterial persistence and resistance through unrelated mechanisms (Lewis 2007). Persisters in biofilms seem to be the main reason for recalcitrant infections. Some of the reasons why biofilms are so difficult to eradicate are restricted penetration of antimicrobials, decreased growth rate and expression of possible biofilm-specific resistance genes. But these reasons do not explain solely the tolerance of biofilms. As Lewis and his group observed in their study, persisters are ultimately responsible for the resistance of the biofilm to killing (Brooun et al. 2000; Keren et al. 2004). It was observed that a small subpopulation survived the antibiotic treatment, and killing did not increase with antibiotic concentration after certain point. Most cells in a biofilm are not necessarily more resistant to killing than planktonic cells and die rapidly when treated with a bactericidal antibiotic that can kill

also slowly growing cells (Lewis 2001). Persisters survive this treatment and are actually preserved by the presence of an antibiotic that inhibits their growth. Paradoxically, the antibiotic aids persisters to persevere.

This bi-phasic killing has been observed previously also, but the role of persisters in biofilm resistance had not been considered in literature before 2000 (Brooun et al. 2000; Lewis 2001). The antibiotics developed since the 40's have been effective against previously life-threatening acute infections, but there are no specific treatments for biofilm infections even today. Now biofilm caused infections are treated the same way as the infections caused by planktonic microbes (Donlan and Costerton 2002). Bacteria in biofilms can attach to practically any surface, on artificial surfaces and on dead or living tissue. Biofilms formed under high shear environments are stronger than on low-shear environments. Despite the clinical relevance, there are currently no viable means for eradicating persisters *in vivo*. However, Kwan and his team (Kwan et al. 2015) have studied an anti-cancer drug, Mitomycin C (MMC), and it seems to be working *in vitro* against persisters at concentrations applied in cancer treatments clinically.

2.3 Biofilm in chronic infections

Bacterial biofilms consist of self-produced matrix protecting the cells attached to a surface and to each other (Bjarnsholt et al. 2009; Costerton et al. 2003). The matrix provides structural stability and protection against threats, like antibiotics and immune cells (Costerton et al. 1999). It was almost four decades ago, when the direct correlation between development of biofilms and persistent infections was suspected. The first to state this notably in the case of *Pseudomonas aeruginosa* colonizing the lungs of CF patients were N. Høiby, J. W. Costerton, and their collaborators (Lam et al. 1980; Lebeaux et al. 2014).

Biofilm cause various diseases in humans, as bacteria are able to attach to practically any surface, such as teeth, heart valves, lungs, middle ear, artificial prosthetics and instruments (Høiby et al. 2010). The presence of biofilms is often associated with chronic infections in clinical settings due to its tolerance toward antimicrobial agents and host immune defence. In contrast, acute bacterial infections are short-term because they involve susceptible, planktonic bacteria, which are rather easily eradicated with

antibiotics together with the host's immune system. It is clear, that classical antibiotic resistance mechanism such as upregulated efflux pumps play small part in the ability of biofilms to tolerate antibiotic treatment (Høiby et al. 2010). Bacteria have many defence mechanisms related to biofilm lifestyle such as producing virulence factors unique to biofilms. For example, it has been established that rhamnolipids produced by *P. aeruginosa* work as defence against polymorphonuclear leukocytes (PMN's) (Jensen et al. 2007).

2.3.1 Chronic wounds

In developed countries as many as 2 % of the population is estimated to develop chronic wounds, a condition associated with reduced quality of life, and high costs to the health care system (Fazli et al. 2009). As growing populations of people suffer from diabetes and cardiovascular diseases, it increases problems with chronic wounds. In these diseases, wounds are formed due to a dysfunction of the venous valves causing hypertension in the veins of legs, followed by increased pressure in capillaries and swelling. These wounds are poorly healing, which leads them highly susceptible to bacterial infections. Normally, a wound would heal in four main phases: coagulation, inflammation, cell proliferations and epithelialization. However, the disturbance in the inflammation phase leads to chronic wounds. One major part of the inflammation process is the elevated activity of PMNs, which produce tissue damaging factors, like free oxygen radicals to their surroundings. Other interesting feature is, that the tissue damaging factors produced by PMNs, might provoke the cells in the biofilm to persister mode, making the biofilm in the wound even more difficult to eradicate (Jensen et al. 2007). As the biofilms offers protection from PMNs, they are not able to reach their target inside the biofilm (Bjarnsholt et al. 2008). At least *P. aeruginosa* is known to produce rhamnolipids that offer shield against the activities of host immune cells.

2.3.2 Lung infections in immunocompromised patients

Cystic fibrosis (CF) is a life-limiting genetic disorder, with highest prevalence in Europe, North America, and Australia (Elborn 2016). The disease is caused by mutation in a gene of a chloride-conducting transmembrane channel called the cystic fibrosis transmembrane conductance regulator (CFTR). There are several hundreds of known mutations that cause

CF and the range of types of mutations is wide (Koch and Høiby 1993). CFTR functions as regulator of anion transport and mucociliary clearance in the airways (Elborn 2016). The malfunctioning CFTR causes dehydration in the respiratory epithelium due to decreased secretion and increased reabsorption of electrolytes and water. The secretions covering the respiratory epithelium are thickened and pulmonary clearance is decreased. This leads inevitably to chronic infections and subsequently in local airway inflammation that is harmful to the lungs. Progressive bacterial infection in the lungs leading to respiratory failure is the most common cause of death in CF patients. *Burkholderia* spp. are among *P. aeruginosa* the most common pathogens to cause pulmonary infections in CF patients (Jones et al. 2004; Singh et al. 2000).

There is no curable treatment for CF or the lung infections in CF. Once the CF lung has been colonized, even long-term antibiotic therapy does not eradicate the infection (Singh et al. 2000). The antibiotic resistance of *P. aeruginosa* in the CF lung arises from the innately resistant biofilm form of bacteria. The challenges in treating pulmonary infections of CF patients, is that antibiotics have been developed and are chosen in practise according to their effectiveness against planktonic bacteria, although the bacteria grow in biofilms or aggregates in the lung (Costerton et al. 1999; Lam et al. 1980).

Chronic granulomatous disease (CGD) is a rare immunodeficiency disease resulting from genetic defects in phagocyte NADPH-oxidase, shifting the patient's phagocytes unable to produce the reactive oxygen species (ROS) needed for proper antimicrobial activity. In chronic granulomatous disease *Burkholderia cenocepacia* can infect the lungs of patients and cause life threatening infections (Brackman et al. 2009; Bylund et al. 2005).

2.3.3 Fatal cepacia syndrome

Burkholderia cepacia complex (Bcc) species are notoriously antibiotic resistant and practically impossible to eradicate from CF lung infections (Sokol et al. 2007). In some cases, infection with *B. cenocepacia* results in a rapid deterioration in lung function, characterized by necrotizing pneumonia, bacteraemia and sepsis, referred to as "cepacia syndrome". Pulmonary colonization of *B. cenocepacia* in CF patients can cause fatal cepacia syndrome that accelerates decline in lung functions, and is essentially untreatable (Jones et al. 2004). This condition is different, and more serious than other lung infections

in CF patients, and cepacia syndrome is rare in other than CF patients. The trouble treating this condition comes from the intrinsic resistance of *B. cenocepacia* to antibiotics (Selin et al. 2015).

2.3.4 Infections of indwelling devices

In many medical conditions, it is necessary to implant a medical device inside the body of a patient. As biofilms can thrive on practically any surface, they may grow on indwelling devices also (Costerton et al. 1999). About 2/3 of nosocomial (hospital acquired) infections are associated with some type of implanted medical device (Bryers 2008). Most commonly used indwelling medical devices (IMD) are urinary and intravascular catheters and microbial infections on these devices are common. Microbial infection can also occur in e. g. artificial heart valves, orthopaedic implants, cardiac pacemakers, ocular prostheses, dental implants and contact lenses (Bryers 2008; Donlan and Costerton 2002).

For infections of some uncomplicated and nonsurgical IMD's, such as contact lenses, the trouble is low, and the implant can be easily replaced. However, catheter-related biofilms can cause bloodstream infections leading to bacteraemia with a significant attributed cost and a high probability of secondary complications including infective endocarditis, septic emboli, and stroke (Lynch and Robertson 2008). Biofilms growing on prosthetic joints can cause also serious infections, which are painful for the patient with high risks for complications, expensive and laborious to replace (Barberán 2006). When an IMD, such as artificial joint, is infected with biofilm, it is usually treated with a course of antimicrobials, but often it is not enough to eradicate the infection and the IMD must be replaced surgically. Another case of infection on implanted devices is tissue fillers (Alhede et al. 2014). Tissue fillers such as gels or silicones are extensively used in the cosmetic business. As with other implanted devices, the use of tissue fillers has led to adverse effects in terms of infections. (Bjarnsholt et al. 2009)

2.3.5 Other biofilm related diseases

The microbial colonies in the oral environment are another example of a complex biofilm system. Pathogenic oral biofilms can cause local infections such as dental caries or

periodontitis (Bryers 2008; Marsh 2006). Microbes originating from gingival tissue can enter the blood stream in the event of a disruption, for example in dental procedure. Microbes entering the bloodstream from gingival cavities can cause life-threatening endocarditis (Donlan and Costerton 2002). Endocarditis can cause severe complications, as microcolonies can detach from the biofilm attached to the heart valve and cause infective emboli.

One particularly tolerant and notorious biofilm infection is tuberculosis. It is typically caused by *Mycobacterium tuberculosis* and usually occurs in the lungs, but may affect practically any organ system. The condition is potentially life-threatening, and is usually treated with an extended course of several bactericidal antimicrobials combined (Liippo 2010). It is estimated that 2 billion people worldwide are infected with *M. tuberculosis* and it cause an estimated almost 2 million deaths each year (Tufariello et al. 2003). Primary infection leads to active disease in only a minority of infected people, and in rest cases the infection is contained by the immune system. The presence of granulomas, formed by the host's immune cells, has an influence on the tolerance of tuberculosis against treatment and contributes to the latency. These granulomas have altered microenvironments ideal for persister formation and favour also other states of dormancy. Characteristic of tuberculosis is that it prevails as latent infection at most cases, being non-infective and not causing symptoms, but when host's immune system is perturbed, the reactivation of latent infection can occur.

2.4 *Burkholderia cenocepacia*

The first member of the *Burkholderia* family, *Burkholderia cepacia* was originally known as *Pseudomonas cepacia* (Burkholder WH 1950). *B. cenocepacia* belongs to the *B. cepacia* complex (Bcc) which consist of 20 closely related and phenotypically similar species (Lewis and Torres 2016). These gram-negative, rod-shaped bacteria are obligate aerobes and have diverse ecological niches. Bcc bacteria are equipped appendage pili of at least five different kinds (Goldstein et al. 1995). These adhesive structures are needed for the initial stages of biofilm formation (Coenye 2010). Bcc species are abundantly found in the environment, especially in the soil, and have non-pathogenic interactions with plants (Coenye and Vandamme 2003). Some Bcc species are plant pathogens, but can cause also infections in mammalians. Bcc isolates have been exploited for various

purposes, including biological control of plant pathogens and plant growth promotion. In humans, these opportunistic bacteria cause respiratory tract infections in immunocompromised patients, especially in people suffering from cystic fibrosis, but also people with chronic granulomatous disease (Brackman et al. 2009). Most commonly isolated species from Bcc infections in patients with CF are *B. multivorans* and *B. cenocepacia* (Lipuma 2010). Antimicrobial treatments fail often, because these bacteria form biofilms easily and have intrinsic resistance and tolerance against antibiotics.

Chronic *Burkholderia* infections share some persistence factors and host immune evasion strategies with other persistent bacteria, such as *M. tuberculosis* and *P. aeruginosa* (Coenye 2010). *B. cenocepacia* biofilms are highly resistant to certain disinfectants also, such as hydrogen peroxide (Lefebre and Valvano 2001). There has been identified several different exopolysaccharides produced by Bcc species, that are likely to be involved in persistence and invasiveness. Chlorhexidine exposure activates multiple efflux pump proteins in these bacteria and at least some of the RND efflux pumps are lifestyle-specific to the biofilm state (Coenye et al. 2011).

2.5 Tolerance and resistance

Antibiotic resistance is a well-known phenomenon where microbial populations obtain mutations that render them insusceptible to certain antibiotics. Bacterial persistence is a different phenomenon by mechanism. In persistence, small fraction of bacterial population can survive lethal doses of antimicrobial agents while the population as a whole remains susceptible (Dörr et al. 2009). Resistant microbes can grow in the presence of an elevated level of an antimicrobial, and the strain's minimum inhibitory concentration (MIC) is increased (Lewis 2001). Antimicrobial resistance is a distinct phenomenon when bacteria are able to prevent the interaction of an antibiotic with a target by various mechanisms (Lewis 2010). Essentially, antimicrobial tolerance is a property of dormant cells, surviving antibiotic treatments in the absence of drug resistance mechanisms. The search for treatments of bacterial infections is complicated because of both bacterial resistance and persistence, which are two distinguish phenomena occurring through unrelated mechanisms.

Moyed and his team noticed in the 1980's, that high persistent bacteria strains had the same minimal inhibitory concentration (MIC) value than normal strains, though the persistent bacteria is not killed by antimicrobials. Work of Moyed in the 1980's was forgotten for a while like Biggers on 1940's. High persistent, but not resistant strain can produce 1000-times more persisters, but has the same MIC. Although persisters can form stochastically, especially in static cultures as a result of fluctuations in gene expression, it has been show that persistence is also induced by a variety of environmental factors (Orman and Brynildsen 2013)

Two forms of bacterial dormancy, persistence and viable but non-culturable state are usually described as two different phenomena, but they share some similarities according to Ayrapetyan's team (2015). Both forms of dormancy are highly tolerant against antibiotics, and they are induced by similar environmental cues (Jayaraman 2008). Also, the molecular mechanism that controls persistence or VBNC state seems to be similar (e. g. TA-modules and stringent response). These states of dormancy able to tolerate high-dose antibiotics, they can be induced by common environmental cues and share molecular mechanisms that control dormancy, which implies that they are closely related states and develop through shared mechanisms (Orman and Brynildsen 2013).

2.6 Techniques used in persister and biofilm studies

For decades, bacteria have been studied in shaken cultures and most often in exponential growth, that is, the archetype of the planktonic life form where they are free floating cells. These growing, planktonic cultures have been the basis for antibiotic screenings ignoring the biofilm mode of growth for decades. With the significance of biofilms in chronic infections, tolerance to antibiotics is further challenge for treatment of chronic infections. To understand the biofilm mode of growth in the *in vivo* setting, the biofilm lifestyle of *P. aeruginosa* has been studied in laboratory settings as surface grown biofilms. Assays using microtiter plates in combinations with crystal violet and resazurin have been developed to study surface-attracted biofilm-forming bacteria (Alhede et al. 2014).

The main methodologies to study persisters are still unchanged from the times of their discovery; the susceptible population is eliminated with antimicrobials and the remaining bacteria is studied (Cañas-Duarte et al. 2014; Maisonneuve et al. 2013). To gain most

persister possible, the culture is usually grown until stationary phase. Another method utilized to study persisters is the fluorescence-assisted cell sorting (FACS) (Shah et al. 2006). This method can be used to isolate the dormant cells according to their transcriptional activity. Cells are first genetically modified to express green fluorescent protein, so that the level of fluorescent protein correlates to transcriptional activity. The cells are sorted by the amount of emitted green fluorescence. Dimmer fluorescence equals to an expression profile associated with the dormant persister state, and the cells can be found highly tolerant to antibiotics.

Persisters can be studied by different methods, but caution is advised for making conclusion of results, as artificially induced persisters may not be equal to naturally occurring persisters (Kwan et al. 2013). Dormancy induced artificially should be regarded as persister-like state and distinguish from the dormancy of naturally (i. e., regulated by TA systems) forming persister cells. Persistence can be induced *in vitro* by compounds mimicking the activity of toxins, or generating overexpression of a toxin by an inducible plasmid. Antibiotics can exert an effect relatable to toxin when halting protein synthesis, and are frequently used to isolating persisters.

3 AIMS OF THE STUDY

There are handful of strategies to study persisters in general, but only a fraction of those have been proven to work with *B. cenocepacia*. It's a species that was chosen for the study because of its ability to form easily biofilms and persisters. This bacteria strain is interesting also for its intrinsically tolerant nature and the lack of proper treatments against infections caused by it. The species causes fatal lung infections in CF patients, and there is no treatment for it other than inadequate combination antibiotic treatment and lung transplant.

The aim of the study was to develop an assay for studying persisters in *B. cenocepacia* biofilms, that could be ultimately applicable for chemical screens of anti-persister agents. The growing of this strain was new to the lab, and to develop an screening assay, some basic techniques had to be optimized, including growth conditions optimization and susceptibility testing. To have coherent results, it is important that the assay has high repeatability and robustness. These characteristics are assessed with various statistical parameters like screening window coefficient (*Z'*), signal to background ratio (S/B) and signal to noise ratio (S/N).

The objective was to find a valid method to evaluate the viability of the remaining bacteria (persisters) after a chemical treatment. Several assays were tried out, and one of the methods was demonstrated to be functional. Stainings such as crystal violet and resazurin were used in susceptibility testing, and other techniques such as viable count determination and luminescent assay kit were utilized in developing the screening method in this study. Since the screening method developed was novel, validation was required to prove the concept. With a validated screening method for anti-persisters it would be possible to identify potential compounds from e.g. natural compound library in primary screenings.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacteria strain

The strain mainly used (*Burkholderia cenocepacia* DSM 16553) was obtained from HAMBI collection University of Helsinki (Faculty of Agriculture and Forestry, Division of Microbiology and Biotechnology) and stored as cryostocks in -80 °C in mixture of LB with 20 % glycerol.

4.1.2 Reagents and equipment

Antibiotics (ciprofloxacin, doxycycline and tobramycin), methanol, ethanol, glycerol, crystal violet and resazurin sodium salt were purchased from Sigma-Aldrich (St- Louis, Missouri, USA). Luria-Bertani (LB) broth and LB agar were obtained from LAB M limited (Heywood, UK), Mitomycin C (MMC) was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Phosphate buffered saline (PBS) was acquired from Lonza (Basel, Switzerland) and dehydroabietic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). Nunclon™ Delta Surface polystyrene flat-bottom 96- microwell plates with clear or white bottoms were purchased from Thermo Fisher Scientific (Nunc; Roskilde, Denmark). Absorbance, fluorescence and luminescence measurements were carried out with a Thermo Fisher Varioskan LUX multiplate reader (Vantaa, Finland). ATP stock solution was obtained from BioThema (Handen, Sweden).

4.2 Methods

4.2.1 Cultivating the bacteria

The pre-cultures were grown overnight in 50 ml Falcon tubes using 20 µl of the glycerol stock in 6 ml LB. Tubes were incubated under aerobic conditions in 37 ˚C with 220 rpm aeration. For the exponential growth, the pre-culture was diluted 1:10 and grown for approximately 4 hours until optical density was 0.3 – 0.5 to reach the mid-exponential phase. Absorbance was measured from 200 µl volume of bacterial suspension in a well

of a microplate with Varioskan Lux at wavelength 595 nm. The mid-exponential culture achieved was then further diluted to gain optimal biofilm growth conditions.

For optimal biofilm growth, cultures were then diluted 1:100 and grown overnight in 96-microwell plates in 37 ˚C and 220 rpm aeration. Shorter incubation times for biofilm development have also been described in the literature, but overnight incubation was chosen to ensure proper attachment (Peeters et al. 2009). Biofilms were grown in 200 µl volume per well, or when measuring luminescence using the CellTiter-Glo® assay (see section 4.5.2) 100 µl of bacteria per well was used.

4.2.2 Viable count determination

Quantifying the colony forming units per millilitre (CFU/ml) was done by plating serial dilutions of bacterial culture on a LB agar plate. A sample was taken from the desired culture, and then diluted subsequently ten-fold up to 8 times. Each dilution was plated on a quarter of a LB agar plate in five 10 µl drops and incubated in 37 ˚C (Figure 2). After an appropriate incubation time, usually 48 hours, the colonies from each drop were counted and an average number of colony forming units was calculated for each dilution with distinguishable colonies.

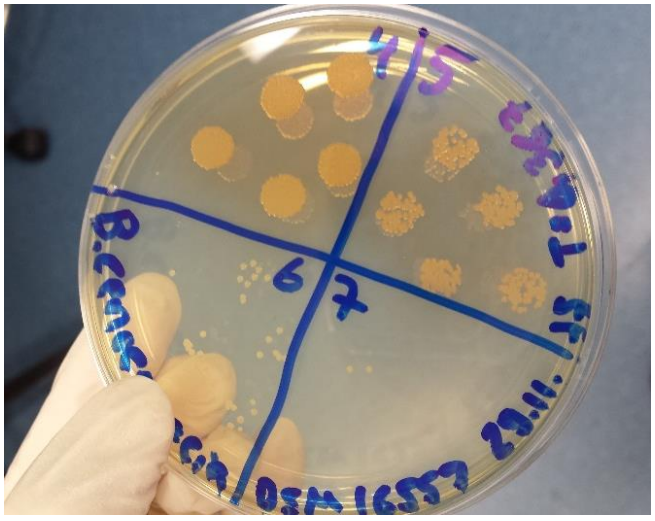

Figure 2. An example of 48-hour old colonies on a LB agar plate for viable count determination.

The count for colony forming units per one millilitre was calculated by dividing the average number of colonies in a drop with the dilution factor of the sample. Only two adjacent countable dilutions were included in the calculations. The formula for viable count determination is shown below.

Viable count = (Average colony count × Dilution factor) / Drop volume.

4.2.3 Quantitation of remaining persisters after treatment

To quantify persisters after treatment, a plating method was carried out according to a protocol by Vandecandelaere et al. (2016). Flat-bottomed, clear 96-microwell plates were used. Biofilms were grown to maturation (see section 4.2.1) in 100 µl volumes and treated with 120 µl of ciprofloxacin and doxycycline (0.1 × MBIC, 4 × MBIC and 10 × MBIC) for 24 hours. After exposure, the antibiotic solutions were removed and wells were washed with PBS and finally 100 µl of PBS was added to each well. The plate was sealed and sonicated for 5 minutes. The cells from 4 replicate wells were transferred to one Eppendorf tube, and sonication was repeated with 100 µl of PBS. These cells obtained with the second sonication were combined with the previous ones in the same Eppendorf tube. The tubes for each sample were centrifuged at 5000 rpm for 10 minutes. Supernatant was removed and 1 ml of PBS was added. Ten-fold serial dilutions were made and drops were plated on LB agar plates as described previously and the number of colonies was calculated.

4.2.4 Resazurin staining

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a redox dye that can be used in biological assays to evaluate the amount of viable bacteria present (Sandberg et al. 2009). Non-fluorescent blue resazurin is reduced to pink highly-fluorescent resorufin when metabolically active bacteria are present. This reaction can be observed visually, or it can be measured spectrophotometrically or the fluorescence can be measured, which was the chosen method in this case due to the high sensitivity of the method. Before measurement the planktonic phase was transferred to a separate plate and the biofilms were washed with PBS to remove any loosely attached bacteria and 200 µl of 0.4 mM resazurin was added in each well on a 96-microplate. When staining planktonic bacteria, it was

transferred to a separate plate and resazurin was used in a final concentration of 20 µM, by addition of 10 µl of the 0.4 mM resazurin stock solution into the bacteria solution. It was observed, that a 2-hour incubation after resazurin addition was needed for sufficient reduction of resazurin in biofilm cultures, planktonic cultures required less exposure time. The plates were incubated with 220 rpm aeration in room temperature in darkness. For evaluating the viability of *B. cenocepacia* cultures, fluorescence was measured with Varioskan LUX at the excitation wavelength of 560 nm and emission wavelength of 590 nm.

4.2.5 CellTiter-Glo® Luminescent Cell Viability Assay

Cell viability was also evaluated with a commercially available CellTiter-Glo® Luminescent Cell Viability Assay kit. With this method, it is possible to measure the level of viable cells in culture, since the level of ATP correlates with the viability of the cells and the method is based on measuring ATP levels. The assay is designed for eukaryotic cells, but it was applied for gram-negative bacterial species of *B. cenocepacia* in this study. Assay was carried out according to the manufacturer's instructions (Promega, 2015).

The bacteria were grown as described earlier, and 100 µl volume of bacterial suspension was used to grow the biofilms on the microwell plates. ATP references for standard curves were always prepared freshly as ten-fold dilution series from the 10 µM stock solution. All luminescence experiments were carried out on white bottomed microwell plates to prevent the signal from leaking to neighbouring wells. This is a simple method to determine the number of viable, metabolically active cells in culture by quantitation of the ATP present. The assay kit is designed for multiwell-plate formats, and it is suitable for high-throughput screening (HTS). The assay is performed by mixing the substrate and buffer components together and adding the mixture in the wells of a 96-mwp containing the cells. Adding the mixed reagent in a cell culture results in cell lysis and generates a stable luminescent signal proportional to the amount of ATP present. The assay is based on the properties of a luciferase enzyme (Ultra-Glo™ Recombinant Luciferase) which creates a luminescent signal when ATP is present (Figure 3). Luminescence measurement was carried at 1000 nm with Varioskan LUX plate reader.

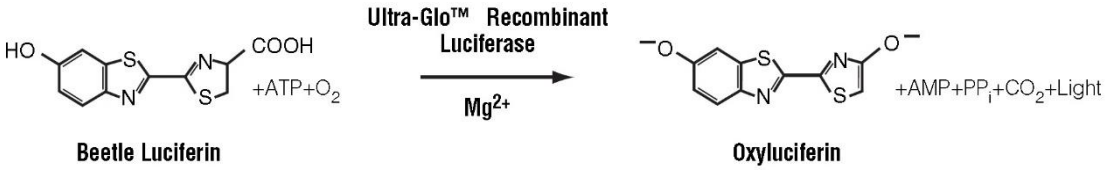

Figure 3. The luciferase reaction. In the presence of ATP, molecular oxygen and Mg+ luciferase catalyses mono-oxygenation of luciferin (CellTiter-Glo® Luminescent Cell Viability Assay protocol, Promega, 2015)

4.2.6 Crystal violet staining

To determine the biomass of the biofilm, crystal violet staining was carried out. The protocol was adapted from the articles Sandberg et al. 2008 and Skogman et al. 2012. 200 µl of bacteria were grown to biofilm as described earlier and after incubation the planktonic phase was removed carefully and the remaining biofilm was washed with MQ-water. The biofilm was fixed with 200 µl of methanol and incubated in room temperature for 15 minutes before methanol was removed and let evaporate to dryness. 190 µl of 1:100 diluted crystal violet was added and let stain for 5 minutes. Stain was removed and washed twice with MQ-water and dried. Finally, the stain was solubilized in 96 % ethanol and incubated for at least one hour before measuring the results at wavelength 595 nm. The assay was carried out in room temperature.

4.2.7 Susceptibility testing

First, susceptibility testing for three antibiotics, doxycycline, ciprofloxacin and tobramycin was carried out. The lowest concentration of antibiotics to inhibit bacterial growth both in planktonic and biofilm forms of the bacteria were determined. All concentrations of antibiotics were prepared in two replicates and all experiments were done twice. Antibiotic stocks were prepared in water (doxycycline, tobramycin) or in dimethyl sulfoxide (DMSO) (ciprofloxacin). The stock solutions were serially diluted 2-fold in LB, to achieve final well concentrations of $1{,}024 – 4.88 \times 10^{-7}$ mg/ml. The bacteria were grown to mid-exponential phase as described earlier. After incubation, this culture suspension was diluted 1:100 to achieve concentration 4×10^6 CFU/ml and then transferred into the microplate with the antibiotics as a total volume of 200 µl per well.

The plates were incubated for 24 hours in 37 °C with 220 rpm aeration. After the incubation, the plates were visually inspected and the planktonic phase was transferred to a clean microplate. Optical density was measured with Varioskan LUX at a wavelength 595 nm to confirm the visual MIC values. To establish minimum biofilm inhibitory concentrations (MBIC) for each antibiotic, resazurin staining and crystal violet staining were carried out. An inhibition percentage was calculated from resazurin experiment for two replicate wells using the formula shown below, percentages over 90 % were considered inhibitory.

$$Inh\text{-}\% = 100 \times (\mu\ Control - \mu\ Sample) / (\mu\ Control - \mu\ Medium).$$

After resazurin staining, biofilm mass was measured with crystal violet. This method measures the biomass of the biofilm, but does not separate the living cells from other biomass.

A post exposure experiment was carried out to see how the antibiotics eradicate mature biofilm. The biofilms were pre-grown, then the antibiotics were added on the biofilm after replacing the planktonic phase with fresh media and the plates were incubated for additionally 24 hours. Resazurin and crystal violet were used subsequently to evaluate the minimum inhibitory concentrations.

A dose-response chart was created for an established anti-persister compound; Mitomycin C (MMC). First the biofilms were grown for 18 hours, and after washing with PBS, MMC was added with fresh media. The mature biofilms were exposed to MMC for 24 hours before using the CellTiter-Glo® assay kit to measure luminescence.

4.2.8 Statistical methods for assay validation

To be able to evaluate the comparability and repeatability of biological assays, statistical parameters like *Z'* (screening window coefficient) together with S/B (signal to background) and S/N (signal to noise) should be calculated (Fallarero et al. 2014). This improves the uniformity of biological assays. Screening window coefficient *Z'* can be used to evaluate the performance of the assay, and it was calculated using formula shown below. *Z'* is measured with controls only, and the value should be 0.5–1.0.

$$Z' = 1 - \frac{3(\sigma\ Control + \ \sigma\ Medium)}{|\mu\ Control + \ \mu\ Medium|}$$

To supplement the *Z'* factor, performance of an assay can be assessed with S/B which should be > 2, and S/N (the higher the better) which measures how robustly maximum and minimum signals can be differentiated. Both values are calculated with control only and the formulas are shown below.

$$\frac{S}{B} = \frac{\mu\ max}{\mu\ min}$$

$$\frac{S}{N} = \frac{\mu\ max - \mu\ min}{\sqrt{SDmax^2 2 + SDmin^2}}$$

Also, to assess the variations between days and plates, CVA% was calculated. To have good repeatability the value should be under 20 %.

$$CVA\ \% = \frac{SD\ max}{\mu\ max - \mu\ min} \times 100\ \%$$

For susceptibility testing, IC50 value was calculated using GraphPad Prism 7 (for mac) and % of inhibition was calculated with the formula presented below.

$$\%\ of\ inhibition\ = \frac{\mu\ max - \mu\ sample}{\mu\ max - \mu\ min} \times 100\ \%$$

5 RESULTS AND DISCUSSION

The goal was to develop a screening method for detecting anti-persister activity in *B. cenocepacia* biofilms. Since the species was new to the lab, the growth conditions had to be optimized before establishing a screening method. Optimizing an assay is challenging and requires major effort. Following the growth optimization, susceptibility testing was carried out for three model antibiotics (ciprofloxacin, doxycycline, tobramycin) as well as MMC, an established anti-persister compound. Dormant persisters are a small fraction of the bacterial population, and a method for detecting even the slightest metabolic activity was needed. The lack of a good assay for persisters was the driving force for this thesis. Several methods were tried out to evaluate the amount of persisters cells, and one of those was deemed as successful for the purpose.

5.1 *Burkholderia cenocepacia* biofilm growth optimization.

The growing of *B. cenocepacia* biofilms is well described in the literature but further optimization of the experimental conditions is typically needed when used for the first time (Buroni et al. 2014; Van Acker et al. 2013). It was observed that bacteria grew quite slowly in narrow 15 ml tubes, and larger 50 ml centrifuge tubes were used for faster initial grow of the bacteria. A wider tube most likely enabled a better oxidation of the culture. After overnight incubation, the pre-culture was diluted 1:10 to achieve exponential growth in four hours. All higher dilutions experimented (1:20, 1:50 and 1:100) took more than four hours to reach the desired turbidity of 0.3 – 0.5. The concentration of bacteria in this optical density was determined with viable count determination, and it contained approximately 4×10^8 CFU/ml.

Different dilutions of mid-exponential culture were experimented for biofilm growing and the biofilm growth was assessed with resazurin and crystal violet staining. Also, the effect of washing the biofilms after planktonic phase removal prior to staining was studied. The dilution of 1:100 resulted in good statistical parameters ($Z' > 0.5$) with or without washing. Although the dilution of 1:10 without washing would give the best Z' value, the washing step was included to ensure an efficient removal of the planktonic bacteria and due to additional reasons, as indicated below. Resazurin staining proved to be a steady method for assessing the viability of the biofilm. For further experiments this

dilution (1:100) of the exponential culture was selected for biofilm growing, and measurement were carried out with resazurin staining with the washing step included (Table 1).

Table 1. Statistics of different conditions tested for biofilm growth. Measurements were carried out with resazurin staining. RSD = Relative standard deviation.

Dilution	wash	Z'	RSD%
1	yes	0.61	10.85
1	no	0.47	16.18
10	yes	0.49	14.02
10	no	0.84	4.63
100	yes	0.66	8.99
100	no	0.52	14.17

The washing step was also included because when studying persisters in further experiments, mainly the persisters in biofilm would be of interest. Persisters are not unique to biofilms, but the planktonic phase contains significantly less persisters (Van Acker et al. 2013). The statistical parameters for the lower dilution (1:10) with no washing were slightly better, because it would contain more bacteria (some planktonic bacteria in addition to the biofilm), but as the planktonic bacteria are not of interest and the biofilm of this concentration was not as well attached as the selected one.

In addition to appropriate temperature and aeration, e. g. concentration and incubation time of the culture affects the properties of the biofilm, like attachment, growth rate and viability. It was also noted, also the volume of the centrifuge tube in initial grow can make a difference in the growth speed. Although the culturing of a certain bacteria is described in the literate, it is always important to optimize the protocol for the lab.

5.2 Cultivating *B. cenocepacia* static cultures.

Because stationary cultures are known to contain more persister cells or cells behaving exactly like persisters, an assay was carried out to achieve stationary phase in *B. cenocepacia* planktonic cultures (Luidalepp et al. 2011; Van Acker et al. 2013). The overnight grown pre-culture was diluted ten-fold and growth was then measured by viable count determination on LB agar plates after 5, 24, 48, 72, 96 and 120 hours of incubation.

Staphylococcus aureus cultures achieve static conditions after an 18-hour incubation (Miettinen et al, unpublished results). The assumption was, that the *B. cenocepacia* culture would enter also a stationary phase after several hours of incubation without any nutrient addition. The culture did grow 2 $\log_{10}$ from the first measurement point (5 hours) to 24 hours and then the number of culturable colonies decreased to starting concentration after 48 hours of incubation (Figure 4). Any plating done after this (72 – 120 hours), did not produce colonies on the LB agar plates.

The static cultures were stained with resazurin at time points 24, 48, 72 and 96 hours to evaluate how metabolically active the cultures were. All measured time points did produce a detectable fluorescence signal with the resazurin dye, except the 96-hour old culture, which had the least metabolic activity as the signal was close to background signal from media. Fluorescence (RFU) describing the metabolic activity measured with resazurin dye was almost the same in 48 and 72-hour old cultures, although the 72-hour culture did not grow at all on the LB agar plates (Figure 4). This phenomenon could be explained by the viable but non culturable (VBNC) state of bacteria. After two days of incubation, it seems that some critical changes in the environmental conditions occur that promotes the bacteria to enter this dormant state within a day. VBNC state can be induced by various environmental factors (Ayrapetyan et al. 2015).

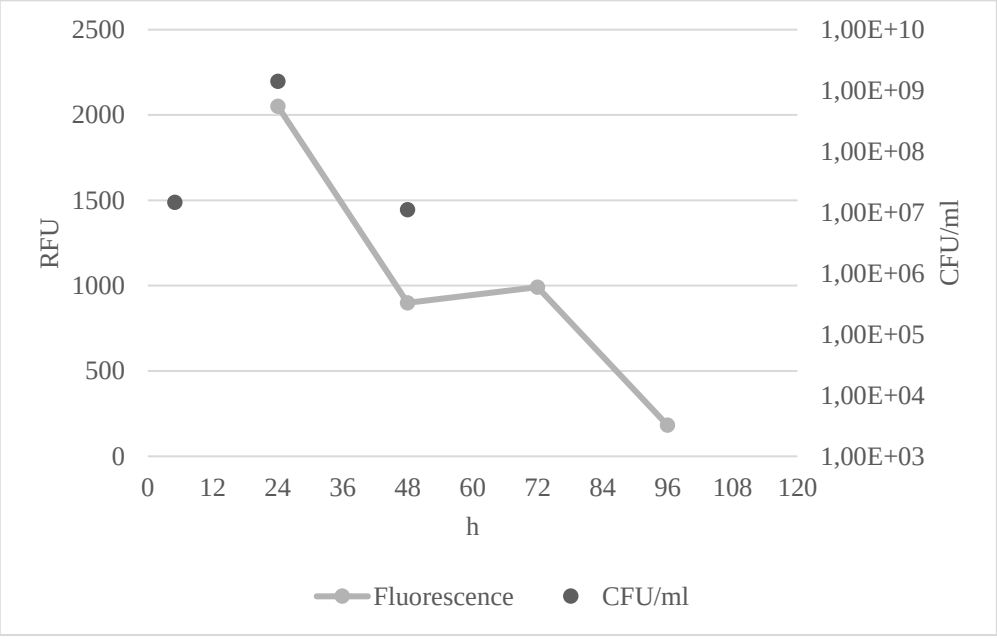

Figure 4. Fluorescence (Relative fluorescence units, RFU) of static cultures and equivalent CFU/ml of the equal cultures.

More accurate growth curve could be possibly obtained with the CellTiter-Glo® Luminescent Cell Viability Assay kit, which can detect even low amount of living bacteria, and the results can be scaled to equivalent number of colony forming units.

5.3 Finding a method for studying anti-persister activity

The aim of the study was to develop a method that would be suitable for anti-persister screenings on *B. cenocepacia* biofilms. The assay developed should be sensitive to low amounts of living bacteria, as persisters are only a small fraction of the population. The assay should also be repeatable and multi-well plate compatible. Several methods were tried out and the results are presented and discussed below. Resazurin staining is a simple protocol for determining the viability of the bacterial culture, but it proved to be too insensitive for detecting small fractions of living bacteria. Second tested method was a protocol by Vandecandelaere (2016), to isolate the persisters from culture by plating method. This however is not suitable for *B. cenocepacia* since non-culturable bacteria may interfere with the results due to long incubation times. Third experiment explored proved to be sensitive to even low number of bacteria and is easy to conduct. Many anti-persister strategies target metabolism activation, like the uptake of antibiotics/aminoglycosides or re-awaking the persisters (Allison et al. 2011).

5.3.1 Evaluating metabolic activity with resazurin

The amount of surviving persisters after treatment was first evaluated using resazurin staining. This assay has been extensively utilized in the laboratory (Skogman et al. 2012), and it was a logical choice to begin with. First, the suitable incubation time for resazurin had to be optimized and 24, 48 and 72 hour old cultures were measured to study the signal development. A 2-hour incubation time was sufficient for resazurin staining of *B. cenocepacia* biofilms (Figure 5). The experiment was carried out with planktonic cells from the static cultures and it was taken into account that planktonic cells stain faster than biofilms.

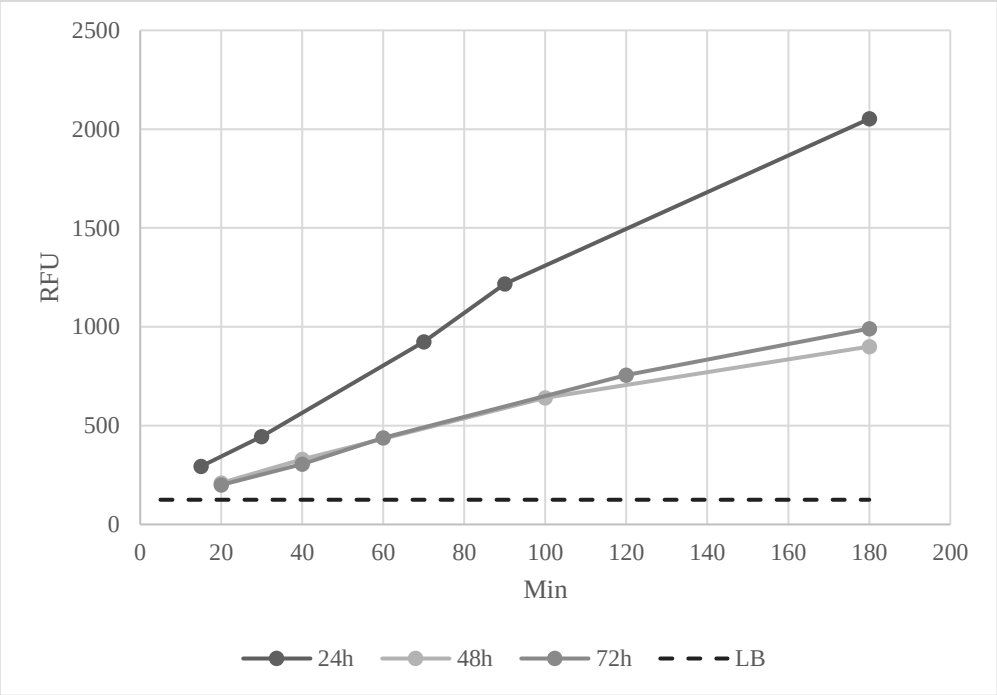

Figure 5. Development of fluorescence signal in resazurin staining of static cultures.

Resazurin staining was carried out to measure the amount of viable bacteria in the culture. Overnight grown biofilms were treated with antibiotics ciprofloxacin and doxycycline at concentrations 0.1, 4 and 10 × MBIC and biofilms were exposed to these substances for 2 hours and 24 hours. This method however did not bring out expected results, as the signal were significantly lower and detection limit for resazurin seems to be relatively high. For *S. aureus*, the detection limit is around 5×10^7 (Sandberg et al. 2009) and it can be assumed that it is at the same magnitude for *B. cenocepacia*.

5.3.2 Quantitation of persisters after treatment by viable count determination

Persister cells were isolated from biofilms grown on microplates and treated with antibiotics according to a previously published protocol for quantifying persisters from biofilms (Vandecandelaere et al. 2016). The viable count determination was conducted to calculate the viable counts (CFU/ml) after treatment. The results of different concentrations of ciprofloxacin and doxycycline (expressed as multiples of MBIC) are seen on Figure 6. A surviving fraction can be seen in 24-hour ciprofloxacin exposure (Figure 6b). However, it is uncertain how much undetectable VBNC bacteria there are.

The result from 24-hour doxycycline exposure show a larger surviving population, which might not all be persisters (Figure 6a). Considering that these bacteria seem to enter the viable but non culturable state, it is not an acceptable method to quantify persister cells. This method required long incubation times and harsh treatment like centrifuging, and it is uncertain how well the cells can tolerate that. There should be a larger difference between the results of different concentrations.

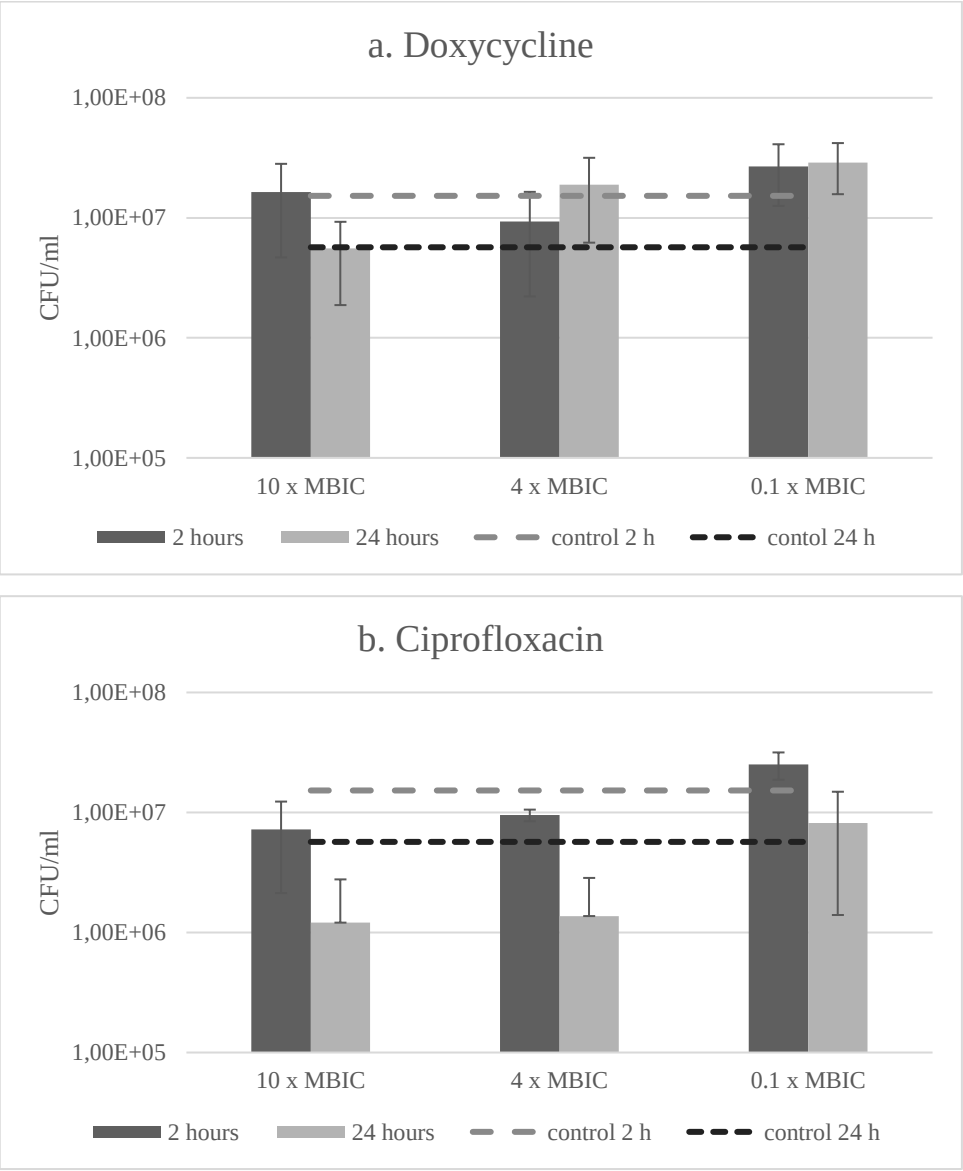

Figure 6. Number of remaining cells (CFU/ml) after a. doxycycline treatment, b. ciprofloxacin treatment

Also, the are a few problems with the protocol as such. The supernatant removal after centrifugation is too vulnerable for pipetting error, since the pellet is not solid and some

bacteria can be aspirated easily. Colony count is not a reliable method for anti-persister screening, because this bacteria strain may enter the VBNC state after 2 days of cultivation, as seen in the attempt to make a growth curve with plating method (Figure 4). This method requires long incubation times and the risk for the bacteria to enter non-culturable state is too high for the results to be reliable. It was also concluded, that ciprofloxacin treatment exposure time should be 24 hours, since the difference between 2-hour and 24-hour exposure is evident.

5.3.3 Evaluating metabolic activity by measuring ATP

CellTiter-Glo® assay kit was utilized to evaluate the amount of ATP present in the treated cultures. The assay is based on the ability of luciferase enzyme to generate a stable luminescence signal when ATP is present in the cultures. This assay was carried out using three different compounds, ciprofloxacin, doxycycline and mitomycin C. The latter one was the most effective compound, as anticipated. The luminescence signal does not by itself tell how many bacteria there is in the culture as in the plating method, but the signal is proportional to the amount of viable bacteria in the culture. To evaluate the number of viable bacteria, a standard curve was calculated from ATP stock solution (Figure 7). Following the standard curve, the bacteria was grown to exponential phase, with known concentration of bacteria without the fear of non-culturable bacteria, and the luminescence of a ten-fold dilution series was measured (Figure 8).

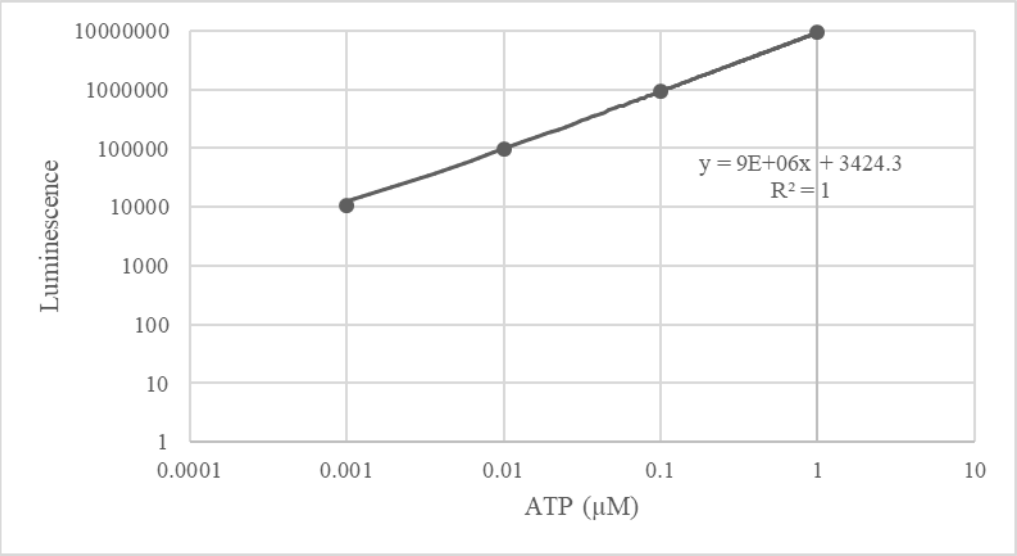

Figure 7. A standard curve for the luminescence signal from known amount of ATP.

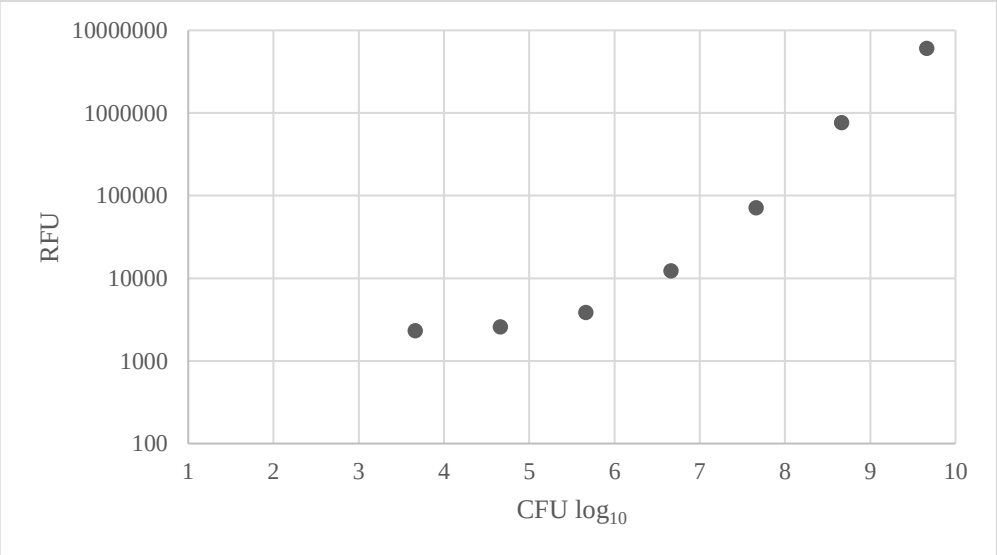

Figure 8. The number of colony forming units corresponding to luminescence signal obtained. Detection limit was determined to be 4.6×10^5 CFU/ml in these assay conditions.

By combining the data from Figure 7 and Figure 8, it is possible to determine the number of viable cells per millilitre in treated cultures (Figure 9). However, the plate count and the luminescence measurement for reference are made with exponentially growing bacteria (Figure 8). This might not be completely comparable to the situation of mature biofilms which have slower metabolism than in the bacteria in exponential phase. As explained earlier in chapter 5.2, the plate counts cannot be made from mature biofilms, as the presence of VBNC is unclear. The data in Figure 9 can be used to evaluate the changes in bacterial concentrations in cultures, and approximate the number of bacteria without plating and counting the colonies. Detection limit (4.6×10^5 CFU/ml) was determined to be the lowest measurement point where S/N was > 2 (2.71).

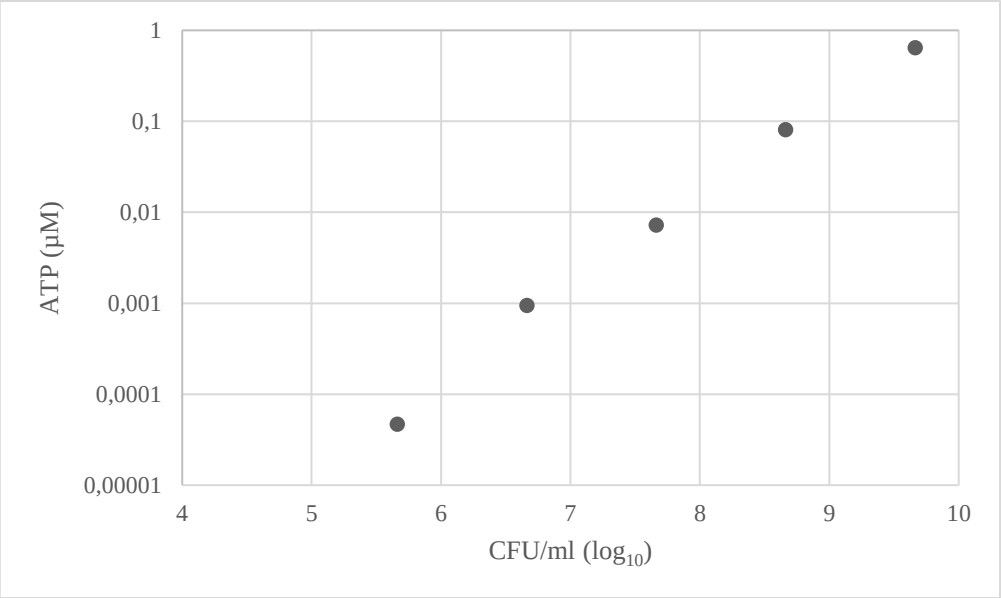

Figure 9. The number of colony forming units can be calculated from the amount of ATP present in the culture. This can be done by measuring the luminescence of cultures and combining the data with the standard curve.

To evaluate performance of the CellTiter-Glo® assay, screening window coefficient *Z'* was calculated with formula shown in chapter 4.2.8. Statistical parameters between plates and days S/N were 8.95 – 12.82, S/B 712 – 775 and Z' 0.66 – 0.77. Assay coefficient variations (CVA %) were 7.80 – 11.17 %. CVA % and Z' can be used to estimate assay performance and variation between days and plates (Fallarero et al. 2014; Iversen et al. 2006).

The best option of the three methods described here turned out to be measuring the amount of ATP present in the treated cultures using CellTiter-Glo®. The method proved to be sensitive, and it can also detect non-culturable cells, the detection limit was 4.6×10^5 CFU/ml for *B. cenocepacia* (Figure 8). With this method, the metabolic activity in the biofilm can be measured and the number of metabolically active cells estimated. When exposing cultures in micro wells to bactericidal concentrations of antibiotic, almost no free-floating bacteria is left and the planktonic phase can be removed before adding the luminescent reagent. Because all living cells contain ATP, culturable cells or not, VBNC bacteria do not interfere with this method. The CellTiter-Glo® assay kit is designed for eukaryotic cells, thus the reliability of the assay in gram-negative bacteria had to be assessed. CellTiter-Glo® assay kit was selected for availability reasons,

although there is also available a similar assay kit suitable for bacteria (BacTiter-Glo®). The reagent in the CellTiter-Glo® assay was observed to lyse gram-negative bacteria and give a high signal although it is designed for eukaryotic cells. This is a sensitive, repeatable method that can detect alive persister cells from cultures treated with bactericidal concentrations of antibiotics.

5.4 Susceptibility testing

5.4.1 Determining minimum inhibitory concentrations for antibiotics

To study persisters alone, they have to be isolated from the normal population. This can be done by killing the non-dormant population with bactericidal concentration of an antibiotic (Keren et al. 2004). Only dormant, non-dividing persister cells survive the bactericidal concentrations of antibiotics. In order to know this concentration, MIC (minimum inhibitory concentration) and MBIC (minimum biofilm inhibitory concentration) values were determined for doxycycline, ciprofloxacin and tobramycin. Antibiotics (tobramycin and ciprofloxacin) were chosen from previous studies found in the literature (Buroni et al. 2014; Van Acker et al. 2013)

Susceptibility testing was done using turbidity measurement, resazurin staining and crystal violet staining. All experiments were done twice, and the resazurin staining method was chosen for the calculations of the MBIC values (Figure 10). Biofilms were grown in the presence of antibiotics for 24 hours before measurement and inhibition values of 90 % or more were considered inhibitory. MBIC for doxycycline, ciprofloxacin and tobramycin were 16 µg/ml, 8 µg/ml and 256 µg/ml, respectively (n = 2). Determined MBIC values were similar with values found in literature (Buroni et al. 2014; Van Acker et al. 2013).

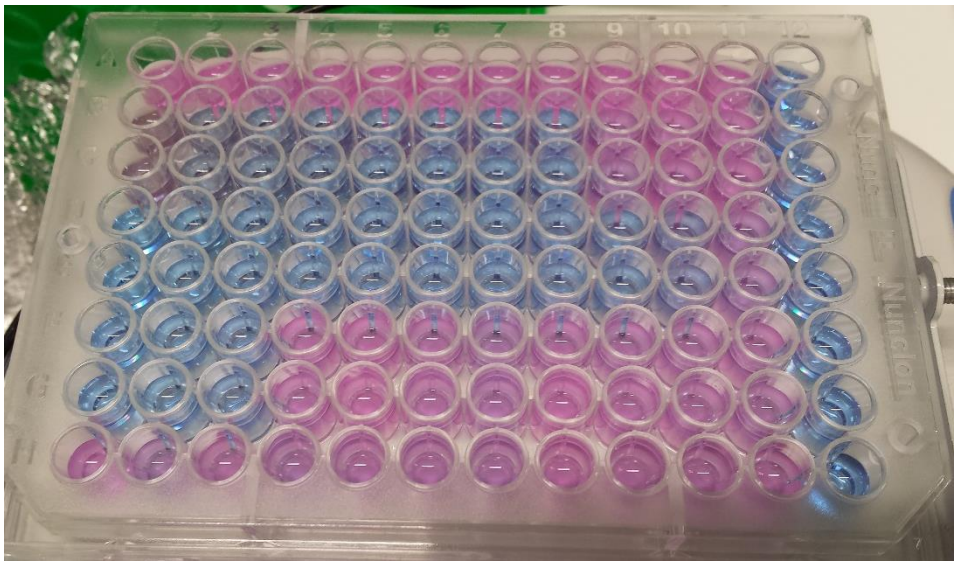

Figure 10. An example of a resazurin stained 96-microplate when determining MBIC. The change of colour is easy to detect even visually. Fluorescence measurement was carried out for results.

The MIC values were evaluated visually and confirmed by measuring the optical density. MIC values for doxycycline, ciprofloxacin and tobramycin were 8 µg/ml, 4 µg/ml and 256 µg/ml respectively (n = 2), results were the same when experiment was repeated, and *Z'* was always above 0.5.

In addition, overnight grown biofilms were treated with antibiotics similarly as in determining MBIC. It is more realistic to expose the mature biofilms to antibiotics when evaluating the efficacy of antibiotics. It is clear that it is more difficult to eradicate a mature biofilm with antibiotic, than preventing biofilm from growing in the presence of an antibiotic. Susceptibility testing is usually done as pre-exposure experiment, but it is not the most realistic way to evaluate the efficacy of an antibiotic. Almost no concentration of antibiotics applied could eradicate the mature biofilm in this experiment. The overnight grown biofilm may slow down the penetration of the antibiotics, and also the dormant persisters that are more abundant in biofilms than in planktonic bacteria, are not killed by the antibiotic. Many antibiotics corrupt the proteins produced by a metabolically active cell, thus dormant cells are not affected by bactericidal antibiotics (Lewis 2010). Resistant bacteria block the antibiotic from binding, but tolerant bacteria prevent the cascade of event leading to corrupted proteins from happening.

5.4.2 Antibiotic treatment of mature biofilms

The effect of two antibiotics against overnight grown biofilms were assessed using CellTiter-Glo® luminescent assay kit. Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic, which inhibits bacterial DNA replication, more specifically the ligase step of the DNA gyrase and topoisomerase IV resulting in cell lysis (Fisher et al. 1989; Hooper 2002). It was developed for clinical use in the 1980's to treat infections due to gram-negative bacteria and has also limited effect against some gram-positive bacteria. Doxycycline is a half-synthetic broad-spectrum tetracycline antibiotic, which inhibits protein synthesis by binding to bacterial ribosome (Nelson and Levy 2011).

Overnight grown biofilms were treated with ciprofloxacin and doxycycline with different concentrations, and the growth inhibition was assessed using CellTiter-Glo® Luminescent assay. Concentrations of antibiotics were selected according to the susceptibility testing carried out earlier. As seen in Figure 11, doxycycline is unable to eradicate mature biofilm even at high concentrations. However, ciprofloxacin is much more effective, being able to kill most of the biofilms.

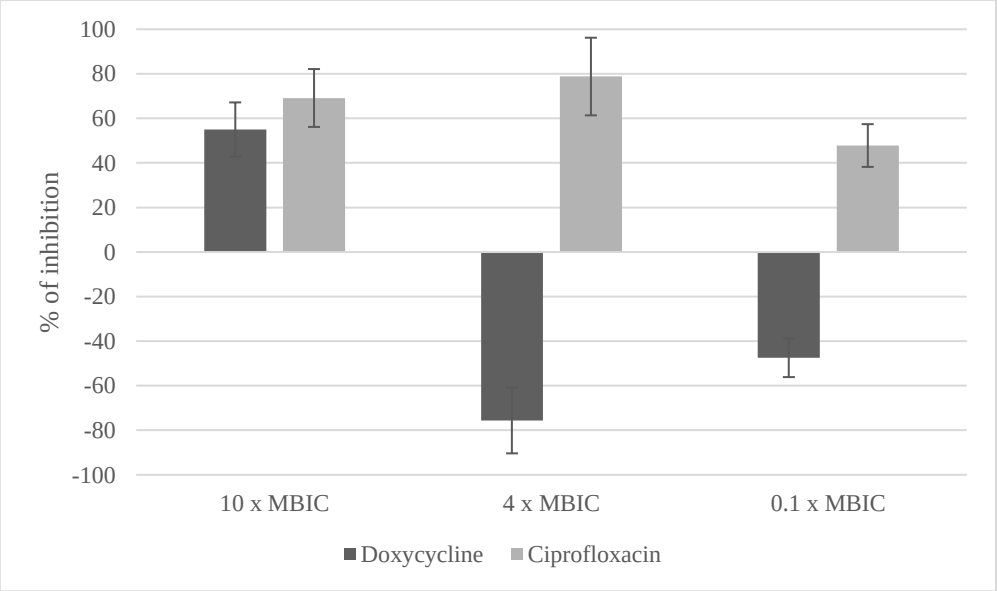

Figure 11. Inhibition-% of different concentrations of doxycycline and ciprofloxacin after 24-hour exposure.

In addition to these antibiotics, the inhibitory effect of another interesting compound, dehydroabietic acid (DHA) was studied. It has shown great activity against *S. aureus*

biofilms (Miettinen, unpublished results), but as expected with gram-negative bacteria, DHA did not have any inhibitory effect on *B. cenocepacia* biofilms even at high concentrations.

5.4.3 Biofilm eradication with Mitomycin C

Mitomycin C (MMC) is an established anti-persisters compound, and the effect against *B. cenocepacia* biofilms was tested with two concentrations (400 µM and 100 µM) using the CellTiter-Glo® assay. The two concentrations tested show high activity against overnight grown biofilms and as there are no published results so far of MMC activity against *B. cenocepacia* biofilms, a killing curve was established (Figure 12).

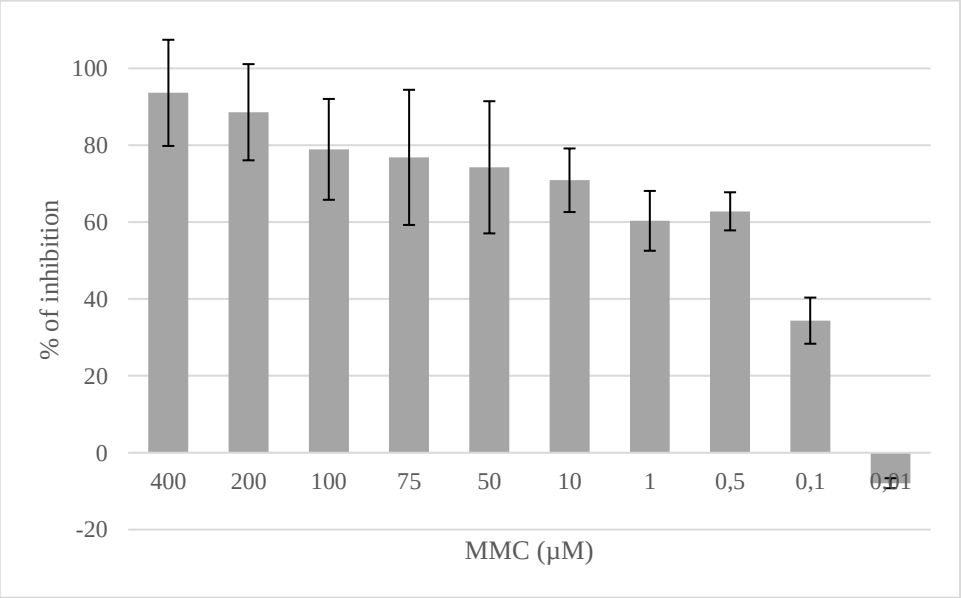

Figure 12. Killing of mature biofilms with Mitomycin C after 24-hour exposure.

The MMC solutions in concentration 400 µM–0.01 µM were prepared in DMSO, so that the final DMSO concentration was always < 5 %, which had no effect on the results. MMC is a chemotherapeutic agent which is known to kill persister cells by damaging the DNA (Kwan et al. 2015). There are no previous results or experiments on MMC eradicating *B. cenocepacia* biofilms. These results prove that MMC kills *B. cenocepacia* biofilms even at very low concentrations. IC_{50} value was calculated to be 0.756 µM (0.183 – 3.13 with 95 % confidence interval). IC_{50} value can be used to evaluate the potency of anti-biofilm effect of the compound.

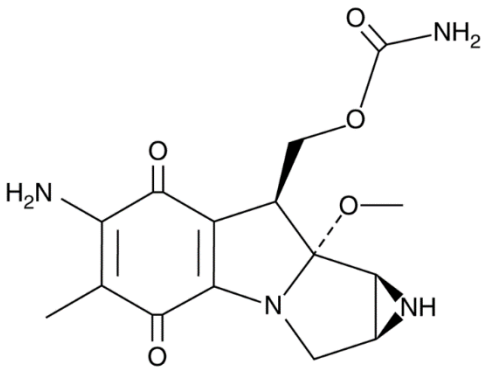

Figure 13. Mitomycin C. (modified from PubChem)

MMC passively diffuses into cells as an amphipathic molecule. When it enters the reducing environment of bacterial cytoplasm, the quinone functional group of the compound is reduced spontaneously initiating cross-linking of two opposing DNA strands. MMC kills non-persister and VBNC cells also (Kwan et al. 2015). Traditional antibiotics (fluoroquinolones, aminoglycosides and β-lactams) are ineffective against persisters cells owing to their mechanism relying on cellular activity. MMC is effective because of its unique mechanism.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Conventional antibiotics are typically not effective against chronic infections and difficulties in the treatment of acute infections are increasing. As the need for new antibiotics is evident, and resistance is evolving faster than the discovery of new antimicrobials, new approaches for counteracting microbial infections should be developed. Persisters have been identified as a main problem in biofilm related infections, and antibiotics targeting growing and dividing cells are unable to kill persisters.

In this thesis, a promising method was developed and validated for detecting anti-persister activity against *B. cenocepacia*. The assay is based on measuring the levels of ATP present in the cultures after treatment and it can be used quantify remaining persisters after treatment using *B. cenocepacia* biofilms. The method is suitable for high-throughput screening and it is sensitive. Otherwise promising method utilizing resazurin staining proved to be too insensitive for detecting small amounts of persister cells. Viable count determination had consistency deficiencies, as the culturability of the bacteria may be affected during the experiment.

Utilizing the method validated, it was confirmed that mitomycin C (MMC) is an effective anti-persister compound against highly tolerant *B. cenocepacia* biofilms even at low concentrations. These bacteria, especially in the biofilm state, are intrinsically tolerant against many antimicrobials and cause severe infections in immunocompromised people. Doxycycline is ineffective against *B. cenocepacia* biofilms, although the bacteria are susceptible to it in planktonic form, and ciprofloxacin is effective at very high concentrations.

The curious bacterial phenotype of persisters needs more investigation and validated methods for the research of anti-persister compounds. Well validated screening methods would able the studies for new anti-persister compound leads and further drug development.

7 REFERENCES

Alhede M, Bjarnsholt T, Givskov M, Alhede M: Chapter One - *Pseudomonas aeruginosa* Biofilms: Mechanisms of Immune Evasion. Advances in Applied Microbiology 86: 1-40, 2014

Alhede M, Kragh KN, Qvortrup K et al.: Phenotypes of Non-Attached *Pseudomonas aeruginosa* Aggregates Resemble Surface Attached Biofilm. PLOS ONE 6: e27943, 2011

Allison KR, Brynildsen MP, Collins JJ: Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473: 216, 2011

Ayrapetyan M, Williams TC, Oliver JD: Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. Trends Microbiol 23: 7-13, 2015

Barberán J: Management of infections of osteoarticular prosthesis. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 12 Suppl 3: 93, 2006

Baugh S, Phillips CR, Ekanayaka AS, Piddock LJV, Webber MA: Inhibition of multidrug efflux as a strategy to prevent biofilm formation. J Antimicrob Chemother 69: 673-681, 2014

Beloin C, Roux A, Ghigo J: Escherichia coli biofilms. Curr Top Microbiol Immunol 322: 249-289, 2008

Bigger J: treatment of staphylococcal infections with penicillin by intermittent sterilisation. The Lancet 244: 497-500, 1944

Bjarnsholt T, Jensen PØ, Burmølle M et al.: Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151: 373-383, 2005

Bjarnsholt T, Jensen PØ, Fiandaca MJ et al.: Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 44: 547-558, 2009

Bjarnsholt T, Kirketerp-Møller K, Jensen PØ et al.: Why chronic wounds will not heal: a novel hypothesis. Wound Repair and Regeneration 16: 2-10, 2008

Brackman G, Hillaert U, Van Calenbergh S, Nelis HJ, Coenye T: Use of quorum sensing inhibitors to interfere with biofilm formation and development in Burkholderia multivorans and Burkholderia cenocepacia. Research in Microbiology 160: 144-151, 2009

Brooun A, Liu S, Lewis K: A dose-response study of antibiotic resistance in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 44: 640-646, 2000

Bryers JD: Medical Biofilms. Biotechnol Bioeng 100: 1-18, 2008

Burkholder WH: Sour kin, a bacterial rot of onion bulbs. Phytoathol 40:115-117, 1950

Buroni S, Matthijs N, Spadaro F et al.: Differential Roles of RND Efflux Pumps in Antimicrobial Drug Resistance of Sessile and Planktonic Burkholderia cenocepacia Cells. Antimicrob Agents Chemother 58: 7424-7429, 2014

Bylund J, Campsall PA, Ma RC, Conway BD, Speert DP: *Burkholderia cenocepacia* Induces Neutrophil Necrosis in Chronic Granulomatous Disease. J Immunol 174: 3562, 2005

Cañas-Duarte SJ., Restrepo S, Pedraza JM: Novel Protocol for Persister Cells Isolation. PLoS ONE 9: e88660, 2014

Chowdhury N, Kwan BW, Wood TK: Persistence Increases in the Absence of the Alarmone Guanosine Tetraphosphate by Reducing Cell Growth. Scientific Reports 6: 20519, 2016

Coenye T: Social interactions in the *Burkholderia cepacia* complex: biofilms and quorum sensing. Future Microbiol 5: 1087-1099, 2010

Coenye T, Van Acker H, Peeters E et al.: Molecular Mechanisms of Chlorhexidine Tolerance in *Burkholderia cenocepacia* Biofilms. Antimicrob Agents Chemother 55: 1912-1919, 2011

Coenye T, Vandamme P: Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ Microbiol 5: 719-729, 2003

Costerton JW, Stewart PS, Greenberg EP: Bacterial Biofilms: A Common Cause of Persistent Infections. Science 284: 1318-1322, 1999

Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G: The application of biofilm science to the study and control of chronic bacterial infections. J Clin Invest 112: 1466-1477, 2003

Cunha M, Sousa S, Leitão JH., Moreira LM, Videira PA, Sá-Correia I: Studies on the Involvement of the Exopolysaccharide Produced by Cystic Fibrosis-Associated Isolates of the *Burkholderia cepacia* Complex in Biofilm Formation and in Persistence of Respiratory Infections. J Clin Microbiol 42: 3052-3058, 2004

Donlan RM: Biofilms: Microbial Life on Surfaces. Emerging Infectious Diseases 8: 881-890, 2002

Donlan RM, Costerton JW: Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clin Microbiol Rev 15: 167-193, 2002

Donne J, Dewilde S: The Challenging World of Biofilm Physiology. Adv Microb Physiol 67: 235-292, 2015

Dörr T, Lewis K, Vulic M: SOS Response Induces Persistence to Fluoroquinolones in *Escherichia coli*. PLOS Genetics 5: e1000760, 2009

Dufour D, Leung V, Lévesque CM: Bacterial biofilm: structure, function, and antimicrobial resistance. Endodontic Topics 22: 2-16, 2010

Elborn JS: Cystic fibrosis. The Lancet 388: 2519-2531, 2016

Fallarero A, Hanski L, Vuorela P: How to Translate a Bioassay Into a Screening Assay for Natural Products: General Considerations and Implementation of Antimicrobial Screens. 2014

Fazli M, Bjarnsholt T, Kirketerp-Møller K et al.: Nonrandom Distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Chronic Wounds. Journal of Clinical Microbiology 47: 4084-4089, 2009

Fisher LM, Lawrence JM, Josty IC, Hopewell R, Margerrison EEC, Cullen ME: Ciprofloxacin and the fluoroquinolones: New concepts on the mechanism of action and resistance. The American Journal of Medicine 87: S2-S8, 1989

Fux CA, Costerton JW, Stewart PS, Stoodley P: Survival strategies of infectious biofilms. Trends Microbiol 13: 34-40, 2005

Galloway WRJD, Hodgkinson JT, Bowden SD, Welch M, Spring DR: Quorum Sensing in Gram-Negative Bacteria: Small-Molecule Modulation of AHL and AI-2 Quorum Sensing Pathways. Chem Rev 111: 28-67, 2011

Gerdes K, Maisonneuve E: Bacterial Persistence and Toxin-Antitoxin Loci. Annu Rev Microbiol 66: 103-123, 2012

Goldstein R, Sun L, Jiang RZ, Sajjan U, Forstner JF, Campanelli C: Structurally variant classes of pilus appendage fibers coexpressed from *Burkholderia* (*Pseudomonas*) *cepacia*. J Bacteriol 177: 1039-1052, 1995

Götz F: Staphylococcus and biofilms. Mol Microbiol 43: 1367-1378, 2002

Gunn JS, Bakaletz LO, Wozniak DJ: What's on the Outside Matters: The Role of the Extracellular Polymeric Substance of Gram-negative Biofilms in Evading Host Immunity and as a Target for Therapeutic Intervention. The Journal of Biological Chemistry 291: 12538-12546, 2016

Hall-Stoodley L, Costerton JW, Stoodley P: Bacterial biofilms: from the Natural environment to infectious diseases. Nat Rev Micro 2: 95-108, 2004

Henrici AT: Studies of freshwater bacteria: I. A direct microscopic technique. J Bacteriol 25, 3: 277-287, 1933

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O: Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35: 322-332, 2010

Hooper DC: Fluoroquinolone resistance among Gram-positive cocci. The Lancet Infectious Diseases 2: 530-538, 2002

Huber B, Riedel K, Hentzer M et al.: The cep quorum-sensing system of Burkholderia cepacia H111 controls biofilm formation and swarming motility. Microbiology-(UK) 147: 2517-2528, 2001

Iversen PW, Eastwood BJ, Sittampalam GS, Cox KL: A Comparison of Assay Performance Measures in Screening Assays: Signal Window, Z' Factor, and Assay Variability Ratio. J Biomol Screen 11: 247-252, 2006

Jayaraman R: Bacterial persistence: some new insights into an old phenomenon. J Biosci 33: 795-805, 2008

Jensen PØ, Bjarnsholt T, Phipps R et al.: Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by Pseudomonas aeruginosa. Microbiology 153: 1329-1338, 2007

Jones A, Dodd M, Govan J et al.: Burkholderia cenocepacia and Burkholderia multivorans: influence on survival in cystic fibrosis. Thorax 59: 948-951, 2004

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K: Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230: 13-18, 2004

Kim J, Wood TK: Persistent Persister Misperceptions. Frontiers in Microbiology 7: 2134, 2016

Koch C, Høiby N: Pathogenesis of cystic fibrosis. The Lancet 341: 1065-1069, 1993

Korch SB, Henderson TA, Hill TM: Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. Mol Microbiol 50: 1199-1213, 2003

Kvist M, Hancock V, Klemm P: Inactivation of Efflux Pumps Abolishes Bacterial Biofilm Formation. Applied and Environmental Microbiology 74: 7376-7382, 2008

Kwan BW, Chowdhury N, Wood TK: Combatting bacterial infections by killing persister cells with mitomycin C. Environ Microbiol 17: 4406-4414, 2015

Kwan BW, Valenta JA, Benedik MJ, Wood TK: Arrested Protein Synthesis Increases Persister-Like Cell Formation. Antimicrobial Agents and Chemotherapy 57: 1468-1473, 2013

Lam J, Chan R, Lam K, Costerton JW: Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis. Infect Immun 28: 546-556, 1980

Lebeaux D, Ghigo J, Beloin C: Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. Microbiology and Molecular Biology Reviews : MMBR 78: 510-543, 2014

Lefebre M, Valvano M: In vitro resistance of Burkholderia cepacia complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production. Microbiology-(UK) 147: 97-109, 2001

Leid JG, Shirtliff ME, Costerton JW, Stoodley aP: Human Leukocytes Adhere to, Penetrate, and Respond to Staphylococcus aureus Biofilms. Infect Immun 70: 6339-6345, 2002

Lewenza S, Conway B, Greenberg EP, Sokol PA: Quorum Sensing in Burkholderia cepacia: Identification of the LuxRI Homologs CepRI. The Journal of Bacteriology 181: 748, 1999

Lewis ERG, Torres AG: The art of persistence: the secrets to Burkholderia chronic infections. Pathogens and Disease 74: ftw070-ftw070, 2016

Lewis K: Persister Cells. Annu Rev Microbiol 64: 357-372, 2010

Lewis K: Persister cells, dormancy and infectious disease. Nature Reviews Microbiology 5: 48-56, 2007

Lewis K: Riddle of Biofilm Resistance. Antimicrob Agents Chemother 45: 999, 2001

Liippo K: Tuberkuloosi. Duodecim 126(1): 65-73, 2010

Lipuma J: The Changing Microbial Epidemiology in Cystic Fibrosis. Clin Microbiol Rev 23: 299, 2010

Luidalepp H, Jãers A, Kaldalu N, Tenson T: Age of Inoculum Strongly Influences Persister Frequency and Can Mask Effects of Mutations Implicated in Altered Persistence. J Bacteriol 193: 3598-3605, 2011

Lynch AS, Robertson GT: Bacterial and Fungal Biofilm Infections. Annu Rev Med 59: 415-428, 2008

Maisonneuve E, Castro-Camargo M, Gerdes K: (p)ppGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity. Cell 154: 1140-1150, 2013

Maisonneuve E, Gerdes K: Molecular Mechanisms Underlying Bacterial Persisters. Cell 157: 539-548, 2014

Marsh PD: Dental plaque as a biofilm and a microbial community - implications for health and disease. BMC oral health 6 Suppl 1: S14, 2006

Moyed HS, Bertrand KP: hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 155: 768-775, 1983

Nelson ML, Levy SB: The history of the tetracyclines. Ann N Y Acad Sci 1241: 17-32, 2011

Orman MA, Brynildsen MP: Establishment of a method to rapidly assay bacterial persister metabolism. Antimicrob Agents Chemother 57: 4398-4409, 2013

Osbourne DO, Soo VWC, Konieczny I, Wood TK: Polyphosphate, cyclic AMP, guanosine tetraphosphate, and c-di-GMP reduce in vitro Lon activity. Bioengineered 5: 264-268, 2014

O'Toole G, Kaplan HB, Kolter R: Biofilm Formation as Microbial Development. Annu Rev Microbiol 54: 49-79, 2000

Patriquin GM, Banin E, Gilmour C, Tuchman R, Greenberg EP, Poole K: Influence of Quorum Sensing and Iron on Twitching Motility and Biofilm Formation in Pseudomonas aeruginosa. J Bacteriol 190: 662-671, 2007

Peeters E, Nelis HJ, Coenye T: In vitro activity of ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and trimethoprim/sulfamethoxazole against planktonic and sessile Burkholderia cepacia complex bacteria. J Antimicrob Chemother 64: 801-809, 2009

Promega Corporation: CellTiter-Glo® Luminescent Cell viability Assay protocol, 2015

PubChem: Compound Database, 2018 (cited: 4.1.2018). Available online: https://pubchem.ncbi.nlm.nih.gov

Purevdorj B, Costerton JW, Stoodley P: Influence of Hydrodynamics and Cell Signaling on the Structure and Behavior of *Pseudomonas aeruginosa* Biofilms. Appl Environ Microbiol 68: 4457-4464, 2002

Rasko DA, Sperandio V: Anti-virulence strategies to combat bacteria-mediated disease. Nature Reviews Drug Discovery 9: 117, 2010

Rijnaarts HHM, Norde W, Bouwer EJ, Lyklema J, Zehnder AJB: Bacterial Adhesion under Static and Dynamic Conditions. Appl Environ Microbiol 59: 3255-3265, 1993

Sandberg ME, Schellmann D, Brunhofer G et al.: Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. J Microbiol Methods 78: 104-106, 2009

Sandberg M, Määttänen A, Peltonen J, Vuorela PM, Fallarero A: Automating a 96-well microtitre plate model for *Staphylococcus aureus* biofilms: an approach to screening of natural antimicrobial compounds. International Journal of Antimicrobial Agents 32: 233-240, 2008

Schmid N, Pessi G, Deng Y et al.: The AHL- and BDSF-Dependent Quorum Sensing Systems Control Specific and Overlapping Sets of Genes in *Burkholderia cenocepacia* H111. PLOS ONE 7: e49966, 2012

Selin C, Stietz MS, Blanchard JE et al.: A Pipeline for Screening Small Molecules with Growth Inhibitory Activity against Burkholderia cenocepacia. PLOS ONE 10: e0128587, 2015

Shah D, Zhang Z, Khodursky AB, Kaldalu N, Kurg K, Lewis K: Persisters: a distinct physiological state of E. coli. BMC Microbiology 6: 53, 2006

Shiau A, Wu C: The Inhibitory Effect of Staphylococcus epidermidis Slime on the Phagocytosis of Murine Peritoneal Macrophages Is Interferon-Independent. Microbiol Immunol 42: 33-40, 1998

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP: Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762, 2000

Skindersoe ME, Alhede M, Phipps R et al.: Effects of Antibiotics on Quorum Sensing in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52: 3648-3663, 2008

Skogman ME, Vuorela PM, Fallarero A: Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms. J Antibiot 65: 453-459, 2012

Sokol PA, Malott RJ, Riedel K, Eberl L: Communication systems in the genus Burkholderia: global regulators and targets for novel antipathogenic drugs. Future Microbiology 2: 555, 2007

Tufariello JM, Chan J, Flynn JL: Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. The Lancet Infectious Diseases 3: 578-590, 2003

Van Acker H, Sass A, Bazzini S et al.: Biofilm-Grown *Burkholderia cepacia* Complex Cells Survive Antibiotic Treatment by Avoiding Production of Reactive Oxygen Species. PLoS ONE 8: e58943, 2013

Van Acker H, Coenye T: The Role of Efflux and Physiological Adaptation in Biofilm Tolerance and Resistance. J Biol Chem 291: 12565-12572, 2016

Vandecandelaere I, Van Acker H, Coenye T: A Microplate-Based System as In Vitro Model of Biofilm Growth and Quantification. Methods Mol Biol 1333: 53, 2016

Wood TK, Knabel SJ, Kwan BW: Bacterial Persister Cell Formation and Dormancy. Appl Environ Microbiol 79: 7116-7121, 2013

Wu Y, Vulic M, Keren I, Lewis K: Role of Oxidative Stress in Persister Tolerance. Antimicrob Agents Chemother 56: 4922-4926, 2012

Zheng D, Taylor GT, Gyananath G: Influence of laminar flow velocity and nutrient concentration on attachment of marine bacterioplankton. Biofouling 8: 107-120, 1994